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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.

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☐ Additional inventors are being named on the \_\_\_\_\_ separately numbered sheets attached hereto**TITLE OF THE INVENTION (500 characters max)**

SYNTHETIC CHEMOKINES, METHODS OF MANUFACTURE, AND USES

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**ENCLOSED APPLICATION PARTS (check all that apply)**

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Application Data Sheet. See 37 CFR 1.76

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Respectfully submitted,

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**SYNTHETIC CHEMOKINES, METHODS OF MANUFACTURE, AND USES****INTRODUCTION****Technical Field**

The present invention relates to chemokines and their derivatives, methods of their manufacture, and uses thereof.

**Background**

Chemokines are small proteins involved in leukocyte trafficking and various other biological processes. Most chemokines localize and enhance inflammation by inducing chemotaxis and cell activation of different types of inflammatory cells typically present at inflammatory sites. Some chemokines have properties apart from chemotaxis, such as inducing the proliferation and activation of killer cells, modulating growth of haematopoietic progenitor cell types, trafficking of haematopoietic progenitor cells in and out of the bone marrow in inflammatory conditions, angiogenesis and tumor growth. (See, e.g., Baggiolini *et al.*, *Ann. Rev. Immunology* (1997) 15:675-705; Zlotnik *et al.*, *Critical Rev. Immunology* (1999) 19(1):1-4; Wang *et al.*, *J. Immunological Methods* (1998) 220(1-2):1-17; and Moser *et al.*, *Intl. Rev. Immunology* (1998) 16(3-4):323-344).

The amino acid sequence, structure and function of many chemokines are known. Chemokines have molecular masses of about 8-10 kDa and show approximately 20-50 percent sequence homology among each other at the protein level. The proteins also share common tertiary structures. All chemokines possess a number of conserved cysteine residues involved in intramolecular disulfide bond formation, which are utilized to identify and classify chemokines. For instance, chemokines having the first two cysteine residues separated by a single amino acid are called "C-X-C" chemokines (also called "alpha" chemokines). Chemokines having the first two cysteine residues adjacent are called "CC" chemokines (also called "beta" chemokines). The "C" chemokines differ from the other chemokines by the absence of a cysteine residue (also called "gamma" chemokines). The C chemokines show similarity to some members of the CC chemokines but have lost



the first and third cysteine residues that are characteristic of the CC and CXC chemokines. Members of the small group of chemokines with the first two cysteine residues separated by three amino acid are called "CXXXC" chemokines (also called "CX<sub>3</sub>C" or "delta" chemokines). There are subgroups of chemokines as well. For instance, CC chemokines containing two additional conserved cysteine residues are known, and sometimes the term "C6-beta" chemokine is used for this subgroup. Most chemokines identified to date are members of the CC and CXC chemokine classes.

The biological activities of chemokines are mediated by receptors. This includes chemokine-specific receptors as well as receptors with overlapping ligand specificity that bind several different chemokines belonging to either the CC chemokines or the group of CXC chemokines. For instance, the CC chemokine SDF-1 $\alpha$  is specific for the CXCR4 receptor, whereas the CXC chemokine RANTES binds to the CCR1, CCR3 and CCR5 receptors. Another example is the chemokine Eotaxin, which is a ligand for the CCR3 and CKR3 receptors. (See, e.g., Cyster, J.G., *Science* (1999) 286:2098-2102; Ponath *et al.*, *J. Experimental Medicine* (1996) 183(6):2437-2448; Ponath *et al.*, *J. Clinical Investigation* (1996) 97(3):604-12; and Yamada *et al.*, *Biochem. Biophys. Res. Communications* (1997) 231(2): 365-368.

Chemokines have been implicated in important disease pathways, such as asthma, allergic rhinitis, atopic dermatitis, cancer, viral diseases, atheroma/atherosclerosis, rheumatoid arthritis and organ transplant rejection. However, a general problem with many chemokines and their potential use as therapeutics relates to their inherent property of promoting or aggravating leukocyte inflammatory responses and infection. To this end, numerous modifications of chemokines have been made in an attempt to generate antagonist of the corresponding chemokine receptor. A classic and representative example is the situation for RANTES. Under certain conditions, wild type RANTES can enhance inflammation and HIV infection (Gordon *et al.*, *J. Virol.* (1999) 73:684-694; Czaplewski *et al.*, *J. Biol. Chem.* (1999) 274:16077-16084). In contrast, substitutions at positions 26 (E26A) and 66 (E66S) of the RANTES polypeptide chain convert the molecule to its non-inflammatory version and improve its ability to compete with its receptors for HIV (Appay *et al.*, *J. Biol. Chem.* (1999) 274(39):27505-27512).

Moreover, N-terminal modifications of RANTES have been made that result in antagonists that can block HIV-1 infection, including N-terminal truncation [RANTES 9-68], addition of methionine ("Met-RANTES"), aminooxypentane ("AOP-RANTES"), or nonanoyl ("NNY-RANTES") (Arenzana-Seisdedos, *et al.*, *Nature* (1996) 383:400; Mack, *et al.*, *J. Exp. Med.* (1998) 187:1215-1224; Proudfoot, *et al.*, *J. Biol. Chem.* (1996) 271:2599-2603; Wells, *et al.*, WO 96/17935; Simmons, *et al.*, *Science* (1997) 276:276-279; Offord *et al.*, WO 99/11666; and Mosier *et al.*, *J. Virology* (1999) 73(5):3544-3550).

Another problematic characteristic of many wild type chemokines as potential therapeutics is their tendency to aggregate at high concentrations, and promiscuous binding and differential activation of chemokine receptors (Murphy *et al.*, *Pharmacological Rev.* (2000) 51(1):145-176), Rollins, B.J., *Blood* (1997) 90(3):909-928; and Wells *et al.*, *Inflammation Res.* (1999) 48:353-362). Aggregation can be problematic for formulation and in some instances aggravate pathology (Czaplewski *et al.*, *J. Biol. Chem.* (1999) 274(23):16077-16084; Czaplewski *et al.*, "Engineering, Biology, and Clinical Development of hMIP-1 $\alpha$ ," (1999) In: *Chemokines in Disease: Biology and Clinical Research*, Ed., C.A. Herbert, Humana Press Inc., Totwa, NJ; Trkola *et al.*, *J. Virol.* (1999) 73(8):6370-6379; Appay *et al.*, *J. Biol. Chem.* (1999) 274(39):27505-27512; Hunter *et al.*, *Blood* (1995) 86(12):4400-4408; Lord *et al.*, *Blood* (1995) 85(12):3412-3415; Lord *et al.*, *Brit. J. Cancer* (1996) 74:1017-1022). Promiscuous binding is a hallmark of chemokines, and may be less desirable in some therapeutic settings. Nevertheless, chemokines hold significant promise as therapeutics (Murphy *et al.*, *Pharmacological Rev.* (2000) 51(1):145-176), Rollins, B.J., *Blood* (1997) 90(3):909-928; and Wells *et al.*, *Inflammation Res.* (1999) 48:353-362).

Publication WO 00/53223 is representative of much of the large body of chemokine literature in that it reportedly discloses novel chemokines, and reports that antagonists can be made, and that PEG or other water-soluble polymers can be attached. US Patent 6,168,784 discloses the chemokine analog NNY-Rantes and suggests modification of the analog with PEG chains at the C-terminus. Recently, even more potent versions of various chemokines have been made (Offord *et al.*, WO 02/04499), including polymer-modified versions that have a polymer attached at

a position corresponding to the C-terminus, a glycosylation site, an aggregation site, and/or a GAG binding site (Koechendoerfer *et al.*, WO 02/04015). Wilkin *et al.* (*Curr. Opinion Biotech.* (1999) 9:412-426) review chemical synthesis of proteins.

While such approaches have improved the target chemokines as potential therapeutics in some cases, one of the challenges in making chemokines suitable as drugs is increasing potency while improving other drug properties such as pharmacokinetics, receptor-specificity, and reduced aggregation. Also, finding a general strategy and method for making potent chemokine receptor antagonist and their use in the preparation of medicaments for use in prevention and/or treatment of disease is desired. The present invention addresses these and other needs.

### **SUMMARY OF THE INVENTION**

The invention is directed to a synthetic chemokines, methods of their manufacture and uses thereof. The synthetic chemokines of the invention comprise a chemokine polypeptide chain having an N-terminus and a C-terminus, where the chemokine polypeptide chain includes (i) an amino acid sequence and cysteine pattern corresponding to a wild type chemokine, and (ii) a C-terminal truncation relative to the wild type chemokine. The invention also provides synthetic chemokines having a C-terminal truncation in combination with one or more additional modifications relative to the corresponding wild type chemokine, including synthetic chemokines having one or more amino acid changes, as well as those modified with one or more covalently attached polymers, and/or chemical adducts. The invention further provides synthetic chemokines having N-terminal and C-terminal modifications, in combination with one or more additional modifications relative to the corresponding wild type chemokine, such as one or more amino acid changes, as well as those modified with one or more covalently attached polymers, and/or chemical adducts. Compositions comprising the synthetic chemokines of the invention in a substantially pure single oligomeric form, such as a monomer or dimer, also are provided. The invention is further directed to methods of producing the synthetic chemokines of the invention, pharmaceutical compositions that include a synthetic chemokine of the invention, methods of treating a chemokine receptor-

mediated disease state in mammals using a synthetic chemokine of the invention, and kits.

### BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1:** Representative Fast Performance Liquid Chromatography (FPLC) chromatograms of synthetic NK chemokines. Shown are FPLC traces for NK 3 (FIG.1A) and NK 4 (FIG.1B) and peaks corresponding to dimer fraction.

**Figure 2:** Representative C4 High Performance Liquid Chromatography (C4-HPLC) and Size Exclusion HPLC (HPLC-SEC) traces, and the corresponding electrospray mass spectroscopy (ES-MS) chromatograms for synthetic NK chemokines. Shown are C4-HPLC, SEC-HPLC, and ES-MS traces for purified dimer pools of synthetic chemokines NK3 (FIG.2A) and NK 4 (FIG.2B).

**Figure 3:** Representative Sodium Dodecyl Polyacrylamide Gels (SDS-PAGE), and Circular Dichroism (CD) spectra for purified synthetic NK chemokines. Shown are SDS-PAGE gels for various reduced and non-reduced purified dimer of NK 3 (FIG.3A) and NK 4 (FIG.3C), and CD spectra for purified dimer for NK 3 (FIG.3B) and NK 4 (FIG.3D). In the SDS-PAGE gels: FIG.3A, lane 1 molecular weight (MW) standard, lanes 2 (reduced) and 3 (non-reduced) are crude protein pool for NK 3; lanes 4 (reduced) and 5 (non-reduced) are monomer fraction for NK 3; lanes 6 (reduced) and 7 (non-reduced) are dimer fraction for NK 3; lanes 8 (reduced) and 9 (non-reduced) are aggregate fraction for NK 3; and lane 10 (blank). FIG.3C, lane 1, MW standard, lanes 2 (reduced) and 3 (non-reduced) are crude protein pool for NK 4; lanes 4 (reduced) and 5 (non-reduced) are monomer fraction for NK 4; lanes 6 (reduced) and 7 (non-reduced) are dimer fraction for NK 4; lanes 8 (reduced) and 9 (non-reduced) are aggregate fraction for NK 4; lane 10 (blank).

**Figure 4:** Representative Size Exclusion Chromatography-Multi-angle Light Scattering Detection (SEC-MALS) chromatogram for purified synthetic NK chemokines. Shown are SEC-MALS traces for purified NK 3 dimer (FIG.4A), purified NK 4 dimer (FIG.4B), and purified oligomeric aggregate (likely an octomer) for NK control (FIG.4C).



**Figure 5:** Shows histogram depicting human CCR5 single transfectant calcium flux assay for a panel of synthetic NK chemokines and controls.

**Figure 6:** Shows graph of human CCR3 single transfectant calcium flux assay for a panel of synthetic NK chemokines and controls.

**Figure 7:** Shows graph of human CCR1 single transfectant calcium flux assay for a panel of synthetic NK chemokines and controls.

**Figure 8:** Representative pharmacokinetic (PK) profiles in rats comparing plasma concentration in nanograms per milliliter (ng/ml) of synthetic NK chemokines versus time in minutes. Shown are PK profiles for NK 3, NK 10 and NK 11, by either an intravenous (IV) (FIG.8A) or subcutaneous (SC) (FIG.8B) route of administration.

### DESCRIPTION OF SPECIFIC EMBODIMENTS

The present invention relates to a synthetic chemokines, methods of their manufacture and uses thereof. In particular, the invention is directed to synthetic chemokines, and especially to C-terminally modified chemokine molecules. The novel bioactive synthetic chemokines of the present invention modulate the activity of a naturally occurring chemokine receptor as determined by a suitable chemokine bioassay. Such molecules may act by antagonizing one or more properties of a chemokine receptor to which they bind (e.g., inhibiting viral infection, causing receptor down-modulation, causing receptor internalization) and thereby "antagonize" the normal cycle of receptor recycling back to the cell surface. In the context of other biological responses, such molecules can act as agonists of a receptor, e.g., inducing calcium flux, initiating chemotaxis, etc. Thus, the bioactive synthetic chemokines of the present invention can act as antagonists (including partial antagonism), but also may act as agonists (including partial agonists), or mixtures of both. Preferred are molecules that exhibit at least one antagonistic property, i.e., an ability to antagonize one or more biological properties of a chemokine receptor to which they bind (e.g., block or partially block (1) viral infection, (2) chemotaxis, (3) receptor cycling etc.). Such molecules may act by binding to (or engaging), but not activating, a chemokine's receptor, or may mediate their action by other means. Surprisingly, it has been found that truncation of

residues from the C-terminus of chemokines has many beneficial and unexpected properties, including retained activity such as those described above, as well as other properties, including reducing the aggregation of chemokines having this tendency as concentration increases.

## **I. SYNTHETIC CHEMOKINES**

### **A. Synthetic Chemokines of the Present Invention**

The preferred synthetic chemokine molecules of the invention comprise a chemokine polypeptide chain having an N-terminus and a C-terminus, where the chemokine polypeptide chain includes (i) an amino acid sequence and cysteine pattern corresponding to a wild type chemokine, and (ii) a C-terminal truncation relative to the wild type chemokine. The N-terminus includes amino acids of the chemokine polypeptide chain that are N-terminal to the first disulfide forming cysteine of the chemokine polypeptide chain. The C-terminus includes amino acids of the chemokine polypeptide chain that are C-terminal to the last disulfide forming cysteine of the chemokine polypeptide chain.

The C-terminal truncation is preferably C-terminal to the core helix region characteristic of chemokines. By "core helix region" is intended residues of a chemokine polypeptide chain that are C-terminal to the last disulfide forming cysteine of the chemokine polypeptide chain, and are capable of forming an  $\alpha$ -helix. This includes the pendant C-terminal residue bearing a free  $\alpha$ -carboxylate and residues adjacent thereto. A prominent secondary structural feature of all chemokines is the anti-parallel  $\beta$  sheet that forms a sheet floor for the C-terminal  $\alpha$ -helix to lay across. Thus the C-terminal  $\alpha$ -helix is a consistent feature of chemokines that is readily identifiable, for instance by homology modeling and comparison, for example, by comparing primary sequences and/or three-dimensional structures of known chemokines, or predicted structures through molecular replacement and energy minimization algorithms (See e.g., Cytokine Reference, Vol. 1, Ligands, A compendium of cytokines and other mediators of host defense, Eds. J.J. Oppenheim and M. Feldmann, Academic Press, 2001)).

By “chemokine polypeptide chain” is intended a polypeptide chain that is substantially homologous to the polypeptide chain of a naturally occurring wild type chemokine. The chemokine polypeptide chain, the N-terminal amino acid sequence, the C-terminal amino acid sequence, and the first and last disulfide-forming cysteines forming the basis of a synthetic chemokine of the present invention can be readily deduced from the corresponding amino acid sequence of the naturally occurring chemokine, as well as by homology modeling with other chemokines of the same class, such as comparison to the amino acid sequences of the known C, CC, CXC and CXXXC chemokines. By “N-terminal amino acid sequence” is intended the amino acid sequence of the chemokine polypeptide chain that is adjacent and N-terminal to the first disulfide-forming cysteine of the naturally occurring chemokine polypeptide chain. By “C-terminal amino acid sequence” is intended the amino acid sequence of the chemokine polypeptide chain that is adjacent and C-terminal to the last disulfide-forming cysteine of the naturally occurring chemokine polypeptide chain.

**Table 1: General chemokine structural motifs and classification\***

Class	N-term.	Cys	N-loop	Cys	Var.	Cys	C-term.
CC	r1	CC	r2; r3; r4	C	r2; r3; r4	C	r2; r3; r4; r5; r6
CXC	r1	CXC	r2; r3; r4	C	r2; r3; r4	C	r2; r3; r4; r5; r6
CX <sub>3</sub> C	r1	CXXXC	r2; r3; r4	C	r2; r3; r4	C	r2; r3; r4; r5; r6
XC	r1	XC	r2; r3; r4	X	r2; r3; r4	C	r2; r3; r4; r5; r6

\*Key: N-term = N-terminal region; C-term. = C-terminal region; Cys = cysteine pattern, where C is cysteine and X is variable amino acid; N-loop = a loop region; Var. = variable region; r1 = pharmacophore region r2 = receptor specificity region; r3 = GAG binding region; r4 = oligomerization region; r5 = core helix region; r6 = C-terminal tail

The following are examples of known naturally occurring “wild type” chemokines, many of which have been described under different names and thus appear several times: 6CKine, 9E3, ATAC, ABCD-1, ACT-2, ALP, AMAC-1, AMCF-1, AMCF-2, AIF, ANAP, Angie, beta-R1, Beta-Thromboglobulin, BCA-1, BLC, blr-1

ligand, BRAK, C10, CCF18, Ck-beta-6, Ck-beta-8, Ck-beta-8-1, Ck-beta-10, Ck-beta-11, cCAF, CEF-4, CINC, C7, CKA-3, CRG-2, CRG-10, CTAP-3, DC-CK1, ELC, Eotaxin, Eotaxin-2, Exodus-1, Exodus-2, ECIP-1, ENA-78, EDNAP, ENAP, FIC, FDNCF, FINAP, Fractalkine, G26, GDCF, GOS-19-1, GOS-19-2, GOS-19-3, GCF, GCP-2, GCP-2-like, GRO1, GRO2, GRO3, GRO-alpha, GRO-beta, GRO-gamma, H400, HC-11, HC-14, HC-21, HCC-1, HCC-2, HCC-3, HCC-4 H174, Heparin neutralizing protein, Humig, I-309, ILINCK, I-TAC, Ifi10, IL8, IP-9, IP-10, IRH, JE, KC, Lymphotactin, L2G25B, LAG-1, LARC, LCC-1, LD78-alpha, LD78-beta, LD78-gamma, LDCF, LEC, Lkn-1, LMC, LAI, LCF, LA-PF4, LDGF, LDNAP, LIF, LIX, LUCT, Lungkine, LYNAP, Manchester inhibitor, MARC, MCAF, MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, MDC, MIP-1-alpha, MIP-1-beta, MIP-1-delta, MIP-1-gamma, MIP-3, MIP-3-alpha, MIP-3-beta, MIP-4, MIP-5, Monotactin-1, MPIF-1, MPIF-2, MRP-1, MRP-2, M119, MDNAP, MDNCF, Megakaryocyte-stimulatory-factor, MGSA, Mig, MIP-2, mob-1, MOC, MONAP, NC28, NCC-1, NCC-2, NCC-3, NCC-4 N51, NAF, NAP-1, NAP-2, NAP-3, NAP-4, NAP S, NCF, NCP, Neurotactin, Oncostatin A, P16, P500, PARC, pAT464, pAT744, PBP, PBP-like, PBSF, PF4, PF4-like, PF4-ALT, PF4V1, PLF, PPBP, RANTES, SCM-1-alpha, SCI, SCY A26, SLC, SMC-CF, ST38, STCP-1, SDF-1-alpha, SDF-1-beta, TARC, TCA-3, TCA-4, TDCF, TECK, TSG-8, TY5, TCF, TLSF-alpha, TLSF-beta, TPAR-1, TSG-1.

By way of illustration, and not by way of limitation, examples of some of the above-listed wild type chemokine polypeptide chains and their corresponding N-terminal, N-loop and C-terminal amino acid sequences are depicted in **Tables 1** and **2**. In **Table 2**, the standard single letter amino acid code is used. As can be appreciated, additional chemokine polypeptide chains are known and obtainable from many different sources including publicly accessible databases such as the Genome Database (Johns Hopkins University, Maryland USA), Protein Data Bank (Brookhaven National Laboratory & Rutgers University, New Jersey USA), Entrez (National Institutes of Health, Maryland USA), NRL 3D (Pittsburgh Supercomputing Center, Carnegie Mellon University, Pennsylvania USA), CATH (University College London, London, UK), NIH Gopher Server (NIH, Maryland USA), ProLink (Boston University, Massachusetts USA), The Nucleic Acid Database (Rutgers University, New Jersey USA), Genebank (National Library of Medicine, Maryland USA), Expasy (Swiss Institute of Bioinformatics, Geneva Switzerland), and the like. Also, new

chemokines, such as those derived from various gene and protein sequencing programs can be identified by homology and pattern matching following standard techniques known in the art, including databases and associated tools for achieving this purpose.

**Table 2: Amino acid sequences of exemplary human chemokines**

**Rantes**

SPYSSDTTPC CFAYIARPLP RAHIKEYFYT SGKCSNPAVV  
FVTRKNRQVC ANPEKKWVRE YINSLEMS

**MIP-1-beta**

APMGSDPPTA CCFSYTLRKL PRHFVIDYFE TTSLCSQPAV  
VFQTKKGRQV CANPSESWVQ EYVDDLELN

**vMIP-II**

GDTLGASWHR PDKCCLGYQK RPLPQVLLSS WYPTSQLCSK  
PGVIFLTKRG RQVCADKSKD WVKKLMQQLP VTAR

**SCM-1**

GSEVSDKRTC VSLTTQRLPV SRIKTYTITE GSLRAVIFIT  
KRGLKVCADP QATWVRDVVR SMDRKSNTN NMIQTKPTGT  
QQSTNTAVTL TG (SEQ ID NO.2)

**Eotaxin**

GPASVPTTCC FNLANRKIPL QRLESYRRIT SGKCPQKAVI  
FKTKLAKDIC ADPKKKWVQD SMKYLDQKSP TPKP

**I309**

KSMQVPFSRC CFSFAEQEIP LRAILCYRNT SSICSNEGLI  
FKLKRGEAC ALDTVGVVQR HRKMLRHCPS KRK

**MCP-1**

QPDAINAPVT CCYNFTNRKI SVQRLASYRR ITSSKCPKEA  
VIFKTIVAKE ICADPKQKWV QDSMDHLDKQ TQTPKT

**MCP-3**

QPVGINSTTT CCYRFINKKI PKQRLESYRR TTSSHCPREA  
VIFKTKLDKE ICADPTQKWV QDFMKHLDKK TQTPKL

**mMCP-5**

GPDAVSTPVT CCYNVVKQKI HVRKLKSYRR ITSSQCPREA  
VIFRTILDKE ICADPKEKWV KNSINHLDKT SQTFILEPSC LG

**MIP-1 $\alpha$  (CCL3)**

SLAADTPTAC CFSYTSRQIP QNFIADYFET SSQCSKPGVI  
 FLTKRSRQVC ADPSEEWVQK YVSDLELSA

**MIP-1 $\beta$  (CCL4)**

APMGSDPPTA CCFSYTARKL PRNFVVDYVE TSSLCSQPAV  
 VFQTKRSKQV CADPSESQVQ EYVYDLELN

**MIP-3 $\alpha$** 

ASNFDCCLGY TDRILHPKFI VGFTRQLANE GCDINAIIFH  
 TKKKLSVCAN PKQTWVKYIV RLLSKKVKNM

**MIP-3 $\beta$** 

GTNDAEDCCL SVTQKPIPGY IVRNHFHYLLI KDGCVRPAVV  
 FTTLRGRQLC APPDQPWVER IIQRLQRTSA KMKRRSS

**MIP-5 (CCL15)**

QFINDAETEL MMSKLPLENP VVLNSFHFAA DCCTSYISQS  
 IPCSLMKSIF ETSSECSKPG VIFLTCKGRQ VCAKPSGPGV  
 QDCMKKLKPY SI

**vMIP-2**

GDTLGASWHR PDKCCLGYQK RPLPQVLLSS WYPTSQLCSK  
 PGVIFLTCKG RQVCADKSKD WVKKLMQQLP VTAR

**MPIF-1**

RVTKDAETEF MMSKLPLENP VLLDRFHATS ADCCISYTPR  
 SIPCSLLESY FETNSECSKP GVIFLTCKGR RFCANPSDKQ  
 VQVCMRMLKL DTRIKTRKN

**LEC**

QPKVPEWVNT PSTCCLKYVE KVLPRRLVVG YRKALNCHLP  
 AIIIFVTKRNR EVCTNPNDW VQEYIKDPNL PLLPTRNLST  
 VKIITAKNGQ PQLLSQ

**HCC**

TKTESSSRGP YHPSECCFTY TTYKIPRQRI MDYYETNSQC  
 SKPGIVFITK RGHVCTNPS DKWVQDYIKD MKEN

**SLC**

SDGGAQDCCL KYSQRKIPAK VVRSYRKQEP SLGCSIPAIL  
 FLPRKRSQAE LCADPKELWV QQLMQHLDKT PSPQKPAQGC  
 RKDRGASKTG KKGKSGSKGCK RTERSQTPKG P

**MDC**

GPYGANMEDS VCCRDYVRYP LPLRVVKHXY WTSDSCPRPG  
VVLTLFRDKE ICADPRVPWV KMILNKLSQ

**TARC**

ARGTNVGREC CLEYFKGAIP LRKLKTWYQT SEDCSRDAIV  
FVTVQGRAIC SDPNNKRVKN AVKYLQSLER S

**TECK**

QGVFEDCCLA YHYPIGWAVL RRAWTYRIQE VSGSCNLPAA  
IFYLPKRHRK VCGNPKSREV QRAMKLLDAR NKVFAKLHHN  
MQTFQAGPHA VKKLSSGNSK LSSSKFSNPI SSSKRNVSLL  
ISANSGL

**SDF1 $\alpha$** 

KPVSLSYRCP CRFFESHVAR ANVKHLKILN TPACALQIVA  
RLKNNNRQVC IDPKLKWIQE YLEKALN

**IP-10**

VPLSRTVRCT CISISNQPVN PRSLEKLEII PASQFCPRVE  
IIATMKKKGE KRCLNPESKA IKNLLKAVSK EMSKRSP

**IL-8**

AVLPRSAKEL RCQCIKTYSK PFHPKFIKEL RVIESGPHCA  
NTEIIVKLSD GRELCCLDPKE NWVQRVVEKF LKRAENS

**MIG**

TPVVRKGRCS CISTNQGTIH LQSLKDLKQF APSPSCEKIE  
IIATLKNGVQ TCLNPDSADV KELIKKWEKQ VSQKKKQKNG  
KKHQKKKVLK VRKSQRSRQK KTT

**GCP-2**

GPVSAVLTEL RCTCLRVTLR VNPKTIGKLQ VFPAGPQCSK  
VEVVASLKNG KQVCLDPEAP FLKKVIQKIL DSGNKKN

**GRO $\alpha$** 

ASVATELRCQ CLQTLQGIHP KNIQSVNVKS PGPHCAQTEV  
IATLKNGRKA CLNPASPIVK KIIEKMLNSD KSN

**GRO $\beta$** 

APLATELRCQ CLQTLQGIHL KNIQSVKVKS PGPHCAQTEV  
IATLKNGQKA CLNPASPMVK KIIEKMLKNG KSN

**GRO $\gamma$** 

ASVVTELRCQ CLQTLQGIHL KNIQSVNVRS PGPHCAQTEV  
IATLKNGKKA CLNPASPMVQ KIIEKILNKG STN

**FK**

QHHGVTKCNI TCSKMTSKIP VALLIHYQQN QASCGKRAII  
LETRQHRLFC ADPKEQWVKD AMQHLDQRQA ALTRNG

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Of particular interest are synthetic chemokines having a C-terminal truncation that eliminates one or more amino acid residues of an aggregation site relative to the corresponding wild type chemokine. By "aggregation site" is intended residue(s) causing self-association of protein monomers. Most chemokines have the potential to form homodimers, with many capable of forming tetramers, and even larger multimers (Cytokine Reference, Vol. 1, Ligands, A compendium of cytokines and other mediators of host defense, Eds. J.J. Oppenheim and M. Feldmann, Academic Press, 2001). Aggregation sites of chemokines typically are found in chemokines capable of forming dimer and multimer complexes at high concentrations (Cytokine Reference, Vol. 1, Ligands, A compendium of cytokines and other mediators of host defense, Eds. J.J. Oppenheim and M. Feldmann, Academic Press, 2001). For instance, Rantes and MIP1 $\beta$  are classic examples of chemokines that form aggregates through self-association of monomers at high concentration, whereas chemokines such as IL-8, SDF1 $\alpha$  and vMIP do not have this tendency to any significant degree.

One of the present findings of the invention is that one or more residues often associated with aggregation are localized to the C-terminus, are typically polar or charged in nature, and can be eliminated by truncation, without the loss of the desired biological activity. By way of example, aggregation sites have been identified in numerous chemokines through various techniques, such as those described above. For instance, wild type Rantes possesses at least two residues involved in aggregation: the glutamic acids at residue positions 26 and 66 (i.e., Glu26 and Glu66; or E26 and E66), of which E66 is located at the C-terminus of the molecule. As another example, the chemokine MIP1 $\alpha$  has at least two residues involved in aggregation, D26 and E66. Similarly, MIP1 $\beta$  has at two main aggregation sites at positions D27 and E67. In yet another example, MCP-1 and Eotaxin have residues involved in aggregation: residues P8 and D68 of MCP-1; and D66 of Eotaxin. Here again at least one of the residues involved in aggregation are localized to the C-terminus. Comparison of these and other chemokines further



reveal that the aggregation sites typically localize C-terminal to the core helix region of the C-terminus, such as E66 and M67 of Rantes, E66 of MIP1 $\alpha$ , E67 of MIP1 $\beta$ , D68 of MCP-1, or D66 of Eotaxin.

Accordingly, in a preferred embodiment, the synthetic chemokines of the invention have a C-terminal truncation that is C-terminal the core helix region of the corresponding wild type chemokine. As noted above, residues of particular interest in the oligomerization of chemokines are polar or charged in nature, which are abundant at the C-terminal region of chemokines. Consequently, the preferred synthetic chemokines of the invention include a C-terminal truncation that comprises a deletion of one or more amino acid residues having a polar or charged side chain relative to said wild type chemokine, such as arginine, lysine, aspartic acid, and glutamic acid. More preferably, the synthetic chemokines of the invention include a truncation that is C-terminal the core helix region of the corresponding wild type chemokine, and where the C-terminal truncation includes a deletion of one or more amino acid residues having a polar or charged side chain relative to said wild type chemokine.

An advantage of such a C-terminal truncation is that the resulting synthetic chemokine can be prepared in a substantially pure single oligomeric form. It has been found that truncation of one or more amino acid residues of an aggregation site at the C-terminus of the corresponding wild type chemokine is sufficient to substantially eliminate aggregation. Such molecules have significantly improved purification and handling properties, particularly under increasing concentrations of the purified product. Accordingly, the invention is further directed to synthetic chemokines having a C-terminal truncation that are in an oligomeric state consisting substantially of monomer or dimer.

In a preferred embodiment, compositions are provided that comprise a synthetic chemokines having a C-terminal truncation, where the synthetic chemokine is in an oligomeric state consisting substantially of monomer or dimer, and where the maximum concentration of the synthetic chemokine is greater than the corresponding wild type chemokine. For example, typical concentration ranges for the synthetic chemokines of the invention can range, for example, up to about 20 milligrams (mg) per milliliter (20 mg/ml), while retaining an oligomeric state consisting

substantially of monomer or dimer. More preferably, the synthetic chemokines of the invention are provided in a concentration range from about 0.5 mg/ml to 15 mg/ml, and most preferably, from about 1 mg/ml to about 10 mg/ml. Of course the actual concentration ranges for a given synthetic chemokine of the invention can be readily determined for a given condition, for instance, by manipulating the formulation to ascertain the maximum tolerated concentration for maintaining a substantially single oligomeric form.

Aggregation sites, including C-terminal aggregation sites, for other chemokines are identifiable via numerous well known methods, such as homology modeling, alanine scanning and comparison of active compounds at increasing concentrations in solution and monitoring for aggregation, self-association using various techniques known in the art. (See e.g., Czaplewski *et al.*, *J. Biol. Chem.* (1999) 274(23):16077-16084; Czaplewski *et al.*, "Engineering, Biology, and Clinical Development of hMIP-1 $\alpha$ ," (1999) In: Chemokines in Disease: Biology and Clinical Research, Ed., C.A. Herbert, Humana Press Inc., Totwa, NJ; Trkola *et al.*, *J. Virol.* (1999) 73(8):6370-6379; Appay *et al.*, *J. Biol. Chem.* (1999) 274(39):27505-27512; Hunter *et al.*, *Blood* (1995) 86(12):4400-4408; Lord *et al.*, *Blood* (1995) 85(12):3412-3415; Lord *et al.*, *Brit. J. Cancer* (1996) 74:1017-1022). And as noted above, the aggregation sites of many chemokines are known, and the techniques for identifying putative aggregation sites are well known (See also, Cytokine Reference, Vol. 1, Ligands, A compendium of cytokines and other mediators of host defense, Eds. J.J. Oppenheim and M. Feldmann, Academic Press, 2001). Thus aggregation sites of chemokines can be identified from published information, homology modeling, through screening, or a combinations of each. Thus the examples presented herein are illustrative of the invention, and thus are not intended to limit the invention.

The synthetic bioactive chemokines of the invention will preferably further include one or more modifications that impart a desired characteristic of inhibiting or promoting an activity of the corresponding wild type chemokine by a suitable chemokine receptor assay, such as assays for receptor binding, competition, calcium flux, MAP kinase, chemotaxis, down-regulation, viral infection and the like. Preferably, such molecules possess N- and/or C-terminal modifications, and/or modifications in the region joining the N-terminal and C-terminal regions, such as a

covalently attached polymer. More preferably, such synthetic chemokines include one or more N-terminal modifications in combination with a C-terminal modification, and most preferably, modifications at the N-terminal, C-terminal, and in the region joining the N-termini and C-termini.

In particular, synthetic chemokines of the invention may include a chemokine polypeptide chain having a C-terminal truncation and one or more additional amino acid residues that differ from an amino acid residue at a corresponding position in the wild type chemokine. This includes N-terminal capping groups, C-terminal capping groups, and internal modifications. For example, the C-terminus may be capped with an amino acid of the formula  $\text{--NH}_2\text{--CH(R)--C(O)--Z}$ , where R is an amino acid side chain that is the same or different from the side chain of the amino acid in a position corresponding to the wild type chemokine, and Z is a C-terminal capping group (e.g., one or more additional amino acids, a polymer, a chemical adduct, and the like), or a polymer attachment residue. For instance, a preferred C-terminal capping group has the formula  $\text{--NH}_2\text{--CH(R)--C(O)--NH}_2$ , has the advantage of providing a C-terminal amino group that can stabilize local hydrogen bonding, and thus the overall protein. Other examples of capping groups include dyes, fluorescent tags, small molecule drugs, purification or attachment handles such as a histidine polymer (His-tag), fusions with serum albumin, antibody or antibody fragment fusion constructs, aliphatic chains, water-soluble polymers and the like. Additional examples include capping with truncated C-terminus with less than the full complement of amino acids from the C-terminal amino acid sequence that is removed relative to the corresponding wild type chemokine, which for instance, results in a synthetic chemokine bearing a C-terminal frame shift in amino acid sequence or otherwise a deletion of one or more residues relative to the corresponding wild type chemokine. Similarly, synthetic chemokines are provided that have a C-terminal truncation in combination with a N-terminal capping group, or both an N-terminal and a C-terminal capping group. Thus the type of capping can impart advantageous properties to the synthetic chemokine of interest.

In a preferred embodiment, synthetic chemokines of the invention may include a chemokine polypeptide chain having a C-terminal truncation and one or more additional amino acid residues modified with a chemical adduct. Chemical adducts

of particular interest include small molecules and polymers and the like, such as dyes, drugs, lipids, carbohydrates, nucleic acids, detectable markers, metal chelators, toxins, aliphatic chains, and polymers, particularly water-soluble polymers.

In one embodiment of particular interest, synthetic chemokines are provided that include small molecule adducts. Such small molecule adducts are preferably attached to the N- and/or C-terminus of the synthetic chemokine of interest. Such small molecule adducts include groups capable of forming a covalent bond to the N-terminus or C-terminus of an amino acid residue, or a derivatized amino acid residue. The attachment can be through any number of covalent bonds, including, but not limited to, amide, amino, ester, thioester, selenoester, ether, thioether, selenoether, oxime, Schiff-base (non-reduced or reduced) and the like. Inclusion of such groups may aid in protection of the N- and/or C-terminus from proteolytic degradation, and/or stability. They also may aid in improving the synthesis and handling properties, including solubility in aqueous solution and in aiding a particular delivery route for a pharmaceutical preparation and its application to a subject in need thereof. Chemical adducts also may be employed as short capping groups. Examples include, but are not limited to, capping groups such as acyl, amide, and other amino acid capping groups, as well as methyl glycine (betaine), dimethyl glycine and other irregular amino acid analogs, and as well as acids such as succinic acid or other similar acids, depending on the intended end use.

In a preferred embodiment, the synthetic chemokines of the present invention comprise a chemokine polypeptide having (i) a C-terminal truncation; and (ii) a one or more chemical adducts attached to an amino acid residue of the chemokine polypeptide chain. Such molecules are preferably those having a chemical adduct comprising an aliphatic chain attached to the N-terminus, the C-terminus, or both. More preferably, the synthetic chemokines will further include one or more of (iii) one or more amino acid derivatives at the N-terminus; and (iv) a polymer attached to the chemokine polypeptide chain.

In one embodiment, the chemokine polypeptide chain of the C-terminally modified synthetic chemokines of the present invention also include those that are modified at its N- and/or C-terminus with an aliphatic chain or polycyclic, and preferably a hydrophobic aliphatic chain, such as described in Koechendoerfer et al.,

WO 02/04015 (CITE OFFORD). Briefly, suitable hydrophobic aliphatic chains include, but are not limited to, hydrophobic aliphatic chains that are five (C5) to twenty-two (C22) carbons in length. The chain may be unsaturated and/or unbranched, or may have variable degrees of saturation and/or branching. The hydrophobic aliphatic chains have the general formula  $C_n(R_m)-$ , where  $C_n$  is the number of carbons and  $R_m$  is the number of substituent groups selected from hydrogen, alkyl, acyl, aromatic or combination(s) thereof, and  $n$  and  $m$  may be the same or different.

The hydrophobic aliphatic chains are joined to the chemokine polypeptide chain via any suitable covalent linkage. Examples of suitable covalent linkages include, but are not limited to: amide, ketone, aldehyde, ester, ether, thioether, thioester, thiozolidine, oxime, oxizolidine, Schiff-base and Schiff-base type linkages (for example, hydrazide). Chemistries suitable for linkage systems are well known and can be utilized for this purpose (see, for example, ROSE; "Chemistry of Protein Conjugation and Cross-Linking", S.S. Wong, Ed., CRC Press, Inc. (1993); Perspectives in Bioconjugate Chemistry, Claude F. Modres, Ed., ACS (1993)). The linkage unit joining the hydrocarbon chains to the chemokine polypeptide chain can vary substantially, with the proviso that the overall length and space filling of the N-terminal region approximates that of the naturally occurring chemokine. The C-terminal region has been found to be more flexible in this regard, so the overall length and space filling can be varied to a greater extent than with the N-terminal region.

In a preferred embodiment, the hydrophobic aliphatic chain attached to the N-terminus is a hydrocarbon chain five (C5) to ten (C10) carbons in length, and the hydrophobic aliphatic chain attached to the C-terminus is a lipid 12 (C12) to twenty (C20) carbons in length. Examples of the C5-C10 hydrocarbon chains include, but are not limited to:  $-C_5H_{11}$ ,  $-C_5H_9$ ,  $-C_5H_7$ ,  $-C_5H_5$ ,  $-C_5H_3$ ,  $-C_6H_{13}$ ,  $-C_6H_{11}$ ,  $-C_6H_9$ ,  $-C_6H_7$ ,  $-C_6H_5$ ,  $-C_6H_3$ ,  $-C_7H_{15}$ ,  $-C_7H_{13}$ ,  $-C_7H_{11}$ ,  $-C_7H_9$ ,  $-C_7H_7$ ,  $-C_7H_5$ ,  $-C_7H_3$ ,  $-C_8H_{17}$ ,  $-C_8H_{15}$ ,  $-C_8H_{13}$ ,  $-C_8H_{11}$ ,  $-C_8H_9$ ,  $-C_8H_7$ ,  $-C_8H_5$ ,  $-C_8H_3$ ,  $-C_9H_{19}$ ,  $-C_9H_{17}$ ,  $-C_9H_{15}$ ,  $-C_9H_{13}$ ,  $-C_9H_{11}$ ,  $-C_9H_9$ ,  $-C_9H_7$ ,  $-C_9H_5$ ,  $-C_9H_3$ ,  $-C_{10}H_{21}$ ,  $-C_{10}H_{19}$ ,  $-C_{10}H_{17}$ ,  $-C_{10}H_{15}$ ,  $-C_{10}H_{13}$ ,  $-C_{10}H_{11}$ ,  $-C_{10}H_9$ ,  $-C_{10}H_7$ ,  $-C_{10}H_5$ , and  $-C_{10}H_3$ . Suitable lipids include, but are not limited to the fatty acid derived lipids and polycyclic steroid derived lipids. The fatty acids include, but

are not limited to, saturated and unsaturated fatty acids. Examples of saturated fatty acids are lauric acid (C12), myristic acid (C14), palmitic acid (C16), steric acid (C18), and arachidic acid (C20). Examples of unsaturated fatty acids include oleic acid (C18), linoleic acid (C18), linolenic acid (C18), eleosteric acid (C18), and arachidonic acid (C20). The polycyclics include, but are not limited to: aldosterone, cholestanol, cholesterol, cholic acid, coprostanol, corticosterone, cortisone, dehydrocholesterol, desmosterol, digitogenin, ergosterol, estradiol, hydroxycorticosterone, lathosterol, prednisone, pregnenolone, progesterone, testosterone, zymosterol, etc. Generally, the fatty acids are usually joined to the chemokine polypeptide chain through the acid component, thereby yielding an acyl-linked moiety, although other linkages may be employed.

In another preferred embodiment, a hydrophobic aliphatic chain when comprised in a bioactive synthetic chemokine of the invention comprise a C5 to C20 saturated or unsaturated acyl chain, such as nonanoyl, nonenoyl, aminooxypentane, dodecanoyl, myristoyl, palmitate, lauryl, palmitoyl, eicosanoyl, oleoyl, or cholyl. For example, the N-terminal substituent can be nonaoyl or aminooxypentane and the C-terminal substituent can be a saturated or unsaturated fatty acid, preferably a C12-C20 fatty acid, or a polycyclic steroid lipid such as cholesterol.

As noted above, the synthetic chemokines of the present invention may include additional amino acids or other moieties that are added to the polypeptide chain. By "amino acid" or "amino acid residue" is intended to include the 20 genetically coded amino acids, rare or unusual amino acids that are found in nature and any of the non-naturally occurring amino acids, such as irregular amino acids; sometimes referred to as amino acid residues when in the context of a peptide, polypeptide or protein. By "amino acid derivative" is intended a derivative of an amino acid or amino acid-like chemical entity.

In particular, synthetic chemokines of the present invention may include one or more amino acid derivatives, such as described in Koechendoerfer et al., WO 02/04015 (CITE OFFORD). Preferred amino acid derivative have the formula  $-(N-C_nR-CO)-$ , where  $C_n$  is 1-22 carbons, R is hydrogen, alkyl or aromatic, and where N and  $C_n$ , N and R, or  $C_n$  and R can form a cyclic structure. Also, N,  $C_n$  and R can each have one or more hydrogens in its reduced form depending on the amino acid

derivative. The alkyl moiety can be substituted or non-substituted, its can be linear, branched, or cyclic, and may include one or more heteroatoms. The aromatic can be substituted or non-substituted, and include one or more heteroatoms. The amino acid derivatives can be made de novo or obtained from commercial sources (See, e.g., Calbiochem-Novabiochem AG, Switzerland; Advanced Chemtech, Louisville, KY, USA; Lancaster Synthesis, Inc., Windham, NH, USA; Bachem California, Inc., Torrance, CA, USA; Genzyme Corp., Cambridge, MA, USA). Examples of such amino acid derivatives include, but are not limited to, aminoisobutyric acid (Aib), hydroxyproline (Hyp), 1,2,3,4-tetrahydroisoquinoline-3-COOH (Tic), indoline-2-carboxylic acid (indol), 4-difluoro-proline (P(4,4DiF)), L-thiazolidine-4-carboxylic acid (Thz), L-homoproline (HoP), 3,4-dehydro-proline ( $\Delta$ Pro), 3, 4-dihydroxyphenylalanine (F(3,4-DiOH)), pBzl,-3, 4-dihydroxyphenylalanine (F(3,4-DiOH, pBzl)), benzophenone (p-Bz), cyclohexyl-alanine (Cha), 3-(2-naphtyl)-alanine ( $\beta$ Nal), cyclohexyl-glycine (Chg), and phenylglycine (Phg).

In a further embodiment, the synthetic bioactive proteins of the present invention may contain "irregular" amino acid residues. As used herein, the term "irregular amino acid residues" is intended to refer to amino acids that are not encoded by RNA and are not ribosomally installed. In this regard, the present invention permits wide selectability and flexibility in the design and/or construction of synthetic bioactive proteins. Examples of non-ribosomally installed amino acids that may be used in accordance with a present invention include: D-amino acids,  $\beta$ -amino acids, pseudo-glutamate,  $\gamma$ -aminobutyrate, ornithine, homocysteine, N-substituted amino acids (R. Simon *et al.*, Proc. Natl. Acad. Sci. U.S.A. (1992) 89: 9367-71; WO 91/19735 (Bartlett *et al.*), U.S. Patent 5,646,285 (Baindur),  $\alpha$ -aminomethyleneoxy acetic acids (an amino acid-Gly dipeptide isostere), and  $\alpha$ -aminooxy acids, etc. Peptide analogs containing thioamide, vinylogous amide, hydrazino, methyleneoxy, thiomethylene, phosphonamides, oxyamide, hydroxyethylene, reduced amide and substituted reduced amide isosteres and  $\beta$ -sulfonamide(s) may be employed.

In a preferred embodiment, the present invention also provides synthetic chemokines comprising a chemokine polypeptide having (i) a C-terminal truncation; and (ii) a polymer attached to the chemokine polypeptide chain. More preferably, the

synthetic chemokines will further include one or more of the N- and/or C-terminal modifications described above.

Preferred synthetic chemokines of the invention have a polymer at a residue of one or more sites selected from a C-terminal site, an aggregation site, a glycosylation site, and a GAG binding site, as described in WO 02/04014. By "C-terminal site" is intended a residue of a chemokine polypeptide chain that is C-terminal to the C-terminal  $\alpha$ -helix of a chemokine. By "GAG binding site" is intended residues coding for GAG binding; typically residues with primary or secondary amines such as lysine and arginine, and sometimes histidine that forms a positive charge cluster on the surface of a protein. By "glycosylation site" is intended residues coding for enzymatic attachment of carbohydrate (oligosaccharide) chain, such as N-linked and O-linked glycosylation sites. More preferred glycosylation sites are those that occur at the C-terminal region of a chemokine. The N-linked glycosylation sites are the most preferred. The glycosylation sites can be natural site or engineered into target protein.

Residues at these sites can be used for attachment, provided they have a side-chain amenable for polymer attachment (i.e., the side chain of an amino acid bearing a functional group, e.g., lysine, aspartic acid, glutamic acid, cysteine, histidine, etc.). Alternatively, a residue at these sites can be replaced with a different amino acid having a side chain amenable for polymer attachment. Also, the side chains of the genetically encoded amino acids can be chemically modified for polymer attachment, or unnatural amino acids with appropriate side chain functional groups can be employed. The preferred method of attachment employs a combination of peptide synthesis and chemical ligation. For polymer attachment to C-terminal sites, the addition of a linker or spacer to the C-terminal end of the C-terminally truncated chemokine polypeptide chain is necessary, such as a C-terminal capping group. This can be accomplished by providing a functional side chain for coupling a water-soluble polymer to the C-terminus of a chemokine of interest that minimizes the potential impact on function of the original C-terminal group at or near that position.

Polymer attachment to a precursor synthetic chemokine with a polymer of interest at one or more sites that retains the in vitro bioactivity can be achieved as



described in WO 02/04014. In vitro bioactivity is a good criteria for assessing function and standard assays for individual chemokines are well known for this purpose (See e.g., Cytokine Reference, Vol. 1, Ligands, A compendium of cytokines and other mediators of host defense, Eds. J.J. Oppenheim and M. Feldmann, Academic Press, 2001; and Cytokine Reference, Vol. 2, Receptors, A compendium of cytokines and other mediators of host defense, Eds. J.J. Oppenheim and M. Feldmann, Academic Press, 2001). As described above, preferred attachment sites are selected from a residue of the precursor chemokine corresponding to a C-terminal site, an aggregation site, a glycosylation site, and a GAG binding site.

The polymer may be joined to the chemokine polypeptide chain through one or more spacers or linkers, which when present, may include polymer chains or units that are biostable or biodegradable. For example, polymers with repeat linkages have varying degrees of stability under physiological conditions depending on bond lability. Polymers with such bonds can be categorized by their relative rates of hydrolysis under physiological conditions based on known hydrolysis rates of low molecular weight analogs, e.g., from less stable to more stable polycarbonates (-O-C(O)-O-) > polyesters (-C(O)-O-) > polyurethanes (-NH-C(O)-O-) > polyorthoesters (-O-C((OR)(R'))-O-) > polyamides (-C(O)-NH-). Similarly, the linkage systems attaching a water-soluble polymer to a target molecule may be biostable or biodegradable, e.g., from less stable to more stable carbonate (-O-C(O)-O-) > ester (-C(O)-O-) > urethane (-NH-C(O)-O-) > orthoester (-O-C((OR)(R'))-O-) > amide (-C(O)-NH-). These bonds are provided by way of example, and are not intended to limit the types of bonds employable in the polymer chains or linkage systems of the water-soluble polymers of the invention.

The polymer-modified synthetic chemokines of the invention preferably bear polymers that are biocompatible, namely, they are non-toxic to biological systems, and many such polymers are known. Such polymers may be hydrophobic or hydrophilic in nature, biodegradable, non-biodegradable, or a combination thereof. These polymers include, but are not limited to, natural polymers such as collagen, gelatin, cellulose, hyaluronic acid, polysaccharides, and polyamino acids, as well as synthetic polymers such as polyesters, polyorthoesters, polyanhydrides, and the like. Examples of hydrophobic non-degradable polymers include polydimethyl siloxanes,

polyurethanes, polytetrafluoroethylenes, polyethylenes, polyvinyl chlorides, and polymethyl methacrylates. Examples of hydrophilic non-degradable polymers include poly(2-hydroxyethyl methacrylate), polyvinyl alcohol, poly(N-vinyl pyrrolidone), polyalkylenes, polyacrylamide, and co-polymers thereof.

The more preferred polymers are water-soluble. By "water-soluble polymer" is intended a polymer that is soluble in water and has an atomic molecular weight greater than about 1,000 Daltons (Da). The Polymer will preferably have an effective hydrodynamic molecular weight of greater than 5,000 Da, and more preferably about 10,000 to 500,000 Da, and most preferably about 10,000 to 300,000 Da. By "effective hydrodynamic molecular weight" is intended the effective water-solvated size of a polymer chain as determined by aqueous-based size exclusion chromatography (SEC). When the water-soluble polymer contains polymer chains having polyalkylene oxide repeat units, such as ethylene oxide repeat units, it is preferred that each chain have an atomic molecular weight of between about 200 and about 80,000 Da and preferably between about 1,500 and about 42,000 Da, with 2,000 to about 40,000 Da being most preferred. Unless referred to specifically, molecular weight is intended to refer to atomic molecular weight, and may be an average of the species of interest.

The water-soluble polymer component can have a wide range of molecular weight, and polymer subunits. These subunits may include a biological polymer, a synthetic polymer, or a combination thereof. Examples of such water-soluble polymers include: dextran and dextran derivatives, including dextran sulfate, P-amino cross linked dextrin, and carboxymethyl dextrin, cellulose and cellulose derivatives, including methylcellulose and carboxymethyl cellulose, starch and dextrans, and derivatives and hydrolyses of starch, polyalkylene glycol and derivatives thereof, including polyethylene glycol, methoxypolyethylene glycol, polyethylene glycol homopolymers, polypropylene glycol homopolymers, copolymers of ethylene glycol with propylene glycol, wherein said homopolymers and copolymers are unsubstituted or substituted at one end with an alkyl group, heparin and fragments of heparin, polyvinyl alcohol and polyvinyl ethyl ethers, polyvinylpyrrolidone, aspartamide, and polyoxyethylated polyols, with the dextran and dextran derivatives, dextrine and dextrine derivatives. It will be appreciated that

various derivatives of the specifically recited water-soluble polymers are also contemplated.

Water-soluble polymers such as those described above are well known, particularly the polyalkylene oxide based polymers such as polyethylene glycol "PEG" (See. e.g., "Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications", J.M. Harris, Ed., Plenum Press, New York, NY (1992); and "Poly(ethylene glycol) Chemistry and Biological Applications", J.M. Harris and S. Zalipsky, Eds., ACS (1997); and International Patent Applications: WO 90/13540, WO 92/00748, WO 92/16555, WO 94/04193, WO 94/14758, WO 94/17039, WO 94/18247, WO 94/28937, WO 95/11924, WO 96/00080, WO 96/23794, WO 98/07713, WO 98/41562, WO 98/48837, WO 99/30727, WO 99/32134, WO 99/33483, WO 99/53951, WO 01/26692, WO 95/13312, WO 96/21469, WO 97/03106, WO 99/45964, and US Patents Nos. 4,179,337; 5,075,046; 5,089,261; 5,100,992; 5,134,192; 5,166,309; 5,171,264; 5,213,891; 5,219,564; 5,275,838; 5,281,698; 5,298,643; 5,312,808; 5,321,095; 5,324,844; 5,349,001; 5,352,756; 5,405,877; 5,455,027; 5,446,090; 5,470,829; 5,478,805; 5,567,422; 5,605,976; 5,612,460; 5,614,549; 5,618,528; 5,672,662; 5,637,749; 5,643,575; 5,650,388; 5,681,567; 5,686,110; 5,730,990; 5,739,208; 5,756,593; 5,808,096; 5,824,778; 5,824,784; 5,840,900; 5,874,500; 5,880,131; 5,900,461; 5,902,588; 5,919,442; 5,919,455; 5,932,462; 5,965,119; 5,965,566; 5,985,263; 5,990,237; 6,011,042; 6,013,283; 6,077,939; 6,113,906; 6,127,355; 6,177,087; 6,180,095; 6,194,580; 6,214,966).

The more preferred water-soluble polymers comprise as a sequential repeat unit ethylene oxide of the formula:  $-(CH_2-CH_2-O)-$ . Examples of preferred ethylene oxide containing polymers are polyethylene glycol ("PEG"), and polyamide ethylene oxides, such as described in below and WO 00/12587, respectively. Briefly, the polyalkylene oxide and polyamide alkylene oxide containing can be of the classic polyethylene glycol (PEG) form, or incorporated in other polypers such as polyamides. For example, a preferred polyamide-based polymer is a polyamide having a molecular weight greater than about 1,000 Daltons of the formula  $-[C(O)-X-C(O)-NH-Y-NH]_n-$  or  $-[NH-Y-NH-C(O)-X-C(O)]_n-$ , where X and Y are divalent radicals that may be the same or different and may be branched or linear, and n is a

discrete integer from 2- 100, and more preferably from 2 to 50, and where either or both of X and Y comprises a biocompatible, substantially non-antigenic water-soluble repeat unit that may be linear or branched. The most preferred water-soluble repeat unit for the polyamides comprises an ethylene oxide of the formula  $-(CH_2-CH_2-O)-$  or  $-(CH_2-CH_2-O)-$ . The number of such water-soluble repeat units can vary significantly, but the more preferred number of such units is from 2 to 500, 2 to 400, 2 to 300, 2 to 200, 2 to 100, and most preferably 2 to 50. An example of a more preferred embodiment is where one or both of X and Y is selected from:  $-((CH_2)_{n1}-(CH_2-CH_2-O)_{n2}-(CH_2)_{n1})-$  or  $-((CH_2)_{n1}-(O-CH_2-CH_2)_{n2}-(CH_2)_{n1})-$ , where  $n1$  is 1 to 6, 1 to 5, 1 to 4 and most preferably 1 to 3, and where  $n2$  is 2 to 50, 2 to 25, 2 to 15, 2 to 10, 2 to 8, and most preferably 2 to 5. An example of a highly preferred embodiment is where X is  $-(CH_2-CH_2)-$ , and where Y is  $-(CH_2-(CH_2-CH_2-O)_3-CH_2-CH_2-CH_2)-$  or  $-(CH_2-CH_2-CH_2-(O-CH_2-CH_2)_3-CH_2)-$ .

For instance, PEG-based chains are amphiphilic, non-immunogenic and not susceptible to cleavage by proteolytic enzymes. Preparations of materials that have been modified by PEG or PEG-based chains, have reduced immunogenicity and antigenicity. See for example, Abuchowski, *et al*, *J. Biol. Chem.* (1977) 252(11):3578-3581; Tsutsumi, *et al*, *Jpn. J. Cancer Res.* (1994) 85:9-12; Poly(ethylene glycol) Chemistry and Biological Applications, ACS Symposium Series 680, J.M. Harris and S. Zalipsky, Eds., American Chemical Society, 1997; and Poly(ethylene glycol) Chemistry, Biotechnical and Biomedical Applications, Topics in Applied Chemistry, J.M. Harris, Ed., Plenum Press, New York, NY, 1992). PEG also serves to increase the molecular size of the material, to which it is attached, thereby increasing its biological half-life. These beneficial properties of the PEG-modified materials make them very useful in a variety of therapeutic applications. Accordingly, this invention also contemplates improving the pharmacokinetics of the polypeptides of the invention, by the modification or "PEGylation" of the polypeptides at sites that are likely to permit the proteins to retain their intrinsic biological activity.

The grafting of PEG chains or PEG-based chains onto proteins is known. See for example, WO 00/12587. Also see, for example, Zalipsky, U.S. Patent No. 5,122,614, which describes PEG that is converted into its N-succinimide carbonate derivative. Also known are PEG chains modified with reactive groups to facilitate

grafting onto proteins. See for example, Harris, U.S. Patent No. 5,739,208, which describes a PEG derivative that is activated with a sulfone moiety for selective attachment to thiol moieties on molecules and surfaces and Harris, *et al.*, U.S. Patent No. 5,672,662, which discloses active esters of PEG acids that have a single propionic or butanoic acid moiety. This area is extensively reviewed in Zalipsky, *Bioconjugate Chemistry* (1995) 6:150-165. Besides use of PEG, Wright, EP 0 605 963 A2 describes linking reagents that contain water soluble polymers that form a hydrazone linkage with an aldehyde group on a protein. All of the aforementioned references are incorporated herein by reference.

It will be appreciated that synthetic chemokines are provided that can be derived from each of these components (i.e., polymer attachment site, the nature of the polymer, and selected of precursor chemokine for polymer modification) alone or in combination. The polymer attachment sites also may overlap or be one in the same, e.g., aggregation site and GAG site are adjacent or are localized at the same site. Moreover, synthetic chemokines are provided that have two or more polymers attached thereto. For example, the invention also includes bioactive synthetic chemokines that comprise a chemokine polypeptide chain and a water-soluble polymer attached thereto at a first site selected from a GAG site and at a second site selected from an aggregation site and a C-terminal site. This aspect of the invention permits, *inter alia*, one to increase the molecular weight and water-solubility (thus improving circulating half-life and other desirable properties afforded by the water-soluble polymer) while eliminating less desirable properties such as aggregation and particular types of GAG binding at the sites of polymer attachment.

In a preferred embodiment, synthetic chemokines are provided having a C-terminal truncation and a water-soluble polymer attached at an amino acid position in the chemokine polypeptide chain corresponding to a GAG binding site of the corresponding wild type chemokine. By way of example, wild type Rantes possesses at least two major GAG binding sites, which comprises an amino acid corresponding to a residue of Rantes selected from Lys44, Lys45, Arg47, Lys55, Lys56, and Arg59 (i.e., K44, K45, R47, K55, K56, and R59). Thus when the synthetic chemokine is an analog of Rantes, and where a water-soluble polymer is attached at GAG binding site thereof, the GAG binding site comprises an amino acid corresponding to a

residue of Rantes selected from K44, K45, R47, K55, K56, and R59. In a preferred embodiment, a water-soluble polymer is attached at a position corresponding to a residue of Rantes selected from K44, K45, R47, with position K45 being more preferred. In this embodiment, polymer attachment at position 45 has the benefit of reducing binding of the synthetic Rantes chemokine analog to the CCR1 receptor, while substantially retaining binding to CCR5.

As another example, when the synthetic chemokine is an analog of MIP1 $\alpha$ , and where a water-soluble polymer is attached at an GAG binding site thereof, the GAG binding site comprises an amino acid corresponding to a residue of MIP1 $\alpha$  selected from R17, R45, and R47. Similarly, when the synthetic chemokine is an analog of MIP1 $\beta$ , and where a water-soluble polymer is attached at an GAG binding site thereof, such an aggregation site can comprise an amino acid corresponding to a residue of MIP1 $\beta$  selected from positions R18, R45, and R46. In these two examples, the preferred site for polymer attachment is at R45 of MIP1- $\alpha$  and R46 of MIP1 $\beta$ . As with Rantes, these modifications are designed to bias binding of the chemokine analog to CCR5.

In a further example, when the synthetic chemokine is an analog of SDF1- $\alpha$ , and where a water-soluble polymer is attached at an GAG binding site, the GAG binding site comprises an amino acid corresponding to residue K24, H25 and K27 of SDF1- $\alpha$ . Here again residues adjacent to these positions can be exploited for polymer attachment in order to achieve substantially the same result, such as N22 or N30 or N33, particularly N33, and thus is embodied in bioactive synthetic chemokines of the invention having a water-soluble polymer attached at an GAG binding site thereof.

In yet another example, when the synthetic chemokine is an analog of IL-8, and where the water-soluble polymer is attached at a GAG binding site, the GAG site comprises an amino acid corresponding to a residue of IL-8 selected from K20, R60, K64, K67 and R68. The preferred site of polymer attachment for a synthetic analog of IL-8 corresponds to position K64 thereof. Another example is MCP-1, so that where the synthetic chemokine is an analog of MCP-1, and where a water-soluble polymer is attached at a GAG site thereof, the GAG site comprises an amino acid

corresponding to a residue of MCP-1 selected from K58 and H66, with K58 being preferred.

As can be appreciated, GAG binding sites are readily identifiable and are preferred sites for polymer-modification, and can be selected for preferential attachment through routine screening for the desired bioactivity. Bioassays suitable for this purpose are well known and replete in the literature (Cytokine Reference, Vol. 1, Ligands, A compendium of cytokines and other mediators of host defense, Eds. J.J. Oppenheim and M. Feldmann, Academic Press, 2001; see also, e.g., Wells *et al.*, *Inflamm. Res.* (1999) 48:353-3362; Lalani *et al.*, *J. Virol.* (1997) 71:4356-4363; Rot, A., *Eur J Immunol* (1993) 23:303-306; Witt *et al.*, *Curr Biol* (1994) 4:394-400; Hoogewerf *et al.*, *Biochem* (1997) 36:13570-13578; Marquezini *et al.*, *Cardiology* (1995) 86:143-146; Wasty *et al.*, *Diabetologia* (1993) 36:316-322). Kuschert *et al.* (*Biochem.* (1999) 38:12959-12968), Koppman *et al.* (*J. Immunol.* (1999) 163:2120-2127) and Proudfoot *et al.* (*J. Biol. Chem.* (2001) 276(14):10620-10626) report on GAG binding and chemokines.

In particular, the GAG binding sites of chemokines can be identified from published information, homology modeling, through screening, or a combinations of each. Moreover, as at least one of the side chain of a residue involved in GAG binding will be located on the surface of the molecule and away from the N-terminal pharmacophore region, these sites should be generally amenable to attachment of a water-soluble polymer. For instance, the BBXB and BBBXB amino acid sequence motifs, where B represents a basic residue, have been shown to be a common heparin-binding motif for several proteins, including several chemokines. (See, e.g., Cardin *et al.*, *Arteriosclerosis* (1989) 9:21-32; Hileman *et al.*, *Bioessays* (1998) 20(2):156-167; and Proudfoot *et al.*, *J. Biol. Chem.* (2001) 276(14):10620-10626). However, GAG binding sites are not restricted to the BBXB or BBBXB motifs. For example, the GAG binding sites in Rantes (<sup>44</sup>RKNR<sup>47</sup> and <sup>55</sup>KKWVR<sup>59</sup>), SDF-1 (<sup>24</sup>KHLK<sup>27</sup>), MIP-1 $\alpha$  (<sup>45</sup>KRSR<sup>48</sup>) and MIP-1 $\beta$  (<sup>45</sup>KRSK<sup>48</sup>) have a BBXB motif, whereas the main GAG-binding residues in IL-8 (Lys20, Lys64, and Arg68), and MCP-1 (Lys59 and Arg66) are spatially separate, but form a basic charge cluster on the protein surface. Thus the basic charge of these ligands accounts for their heparin-binding properties. GAG sites are also identifiable by alanine scanning of

basic residues (e.g., Lys, His and Arg), NMR and comparison of active compounds in GAG/heparin binding assays, as well as NMR studies adapted for this purpose (See, e.g., Proudfoot *et al.*, *J. Biol. Chem.* (2001) 276(14):10620-10626; Trkola *et al.*, *J. Virol.* (1999) 73(8):6370-6379; Appay *et al.*, *J. Biol. Chem.* (1999) 274(39):27505-27512; Hunter *et al.*, *Blood* (1995) 86(12):4400-4408; Lord *et al.*, *Blood* (1995) 85(12):3412-3415; Lord *et al.*, *Brit. J. Cancer* (1996) 74:1017-1022). And as noted above, the GAG binding sites of many chemokines are known, and the techniques for identifying putative GAG sites are well known (See also, Cytokine Reference, Vol. 1, Ligands, A compendium of cytokines and other mediators of host defense, Eds. J.J. Oppenheim and M. Feldmann, Academic Press, 2001).

In addition to synthetic chemokines having one or more N-terminal, C-terminal and/or polymer modifications, the synthetic chemokines of the present invention also may include one or more amino acid substitutions, insertions or deletions elsewhere in the polypeptide chain, such as described in WO 02/04015. In a preferred embodiment, changes are made in the N-loop of the chemokine polypeptide chain to increase its specificity/selectivity for a target receptor. In this way, the N-loop of the bioactive synthetic chemokine of the present invention may block a specific receptor while minimizing the effect on other of its possible co-receptors. By "N-loop" is intended the 20 to 26 amino acid sequence region adjacent/C-terminal to the first conserved cysteine pattern defining the N-terminal region of a given chemokine polypeptide chain (see, **Tables 1 and 2**). For example, as read in the N- to C-terminal direction of the chemokine polypeptide chain, the N-loop of a CC chemokine is the region of amino acids located between and adjacent/C-terminal to the first and second conserved cysteine amino acids and adjacent/N-terminal to the third conserved cysteine amino acid.

With respect to the chemokine polypeptide chain, the amino acid sequence of this component is substantially homologous to the corresponding naturally occurring wild type molecule. The term "substantially homologous" when used herein includes amino acid sequences having at least 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% sequence homology with the given sequence (95 - 99% preference). This term can include, but is not limited to, amino acid sequences having from 1 to 20, from 1 to 10 or from 1 to 5 single amino acid deletions, insertions or substitutions relative to



a given sequence provided that the resultant polypeptide is capable of binding to at least one of the chemokine receptors of the corresponding wild type chemokine.

For instance, it is well known in the art that certain amino acids can be replaced with others resulting in no substantial change in the properties of a polypeptide, including but not limited to conservative substitutions of amino acids. Such possibilities are within the scope of the present invention. It should also be noted that deletions or insertions of amino acids can often be made which do not substantially change the properties of a polypeptide. The present invention includes such deletions or insertions (which may be, for example up to 10, 20 or 50% of the length of the specific chemokine polypeptide sequence of the corresponding naturally occurring chemokine).

In particular, chemokines may be subjected to substantial modifications, including mixing and matching different chemokine polypeptide segments to create additional diversity, such as the modular 'cross-over' synthesis approach described in WO 99/11655, which reference is incorporated herein in its entirety by reference. Recombinant approaches may also be applied to generate backbone changes. For example, directed evolution techniques, such as phage display or modular shuffling, may be used to generate chemokines with increased receptor specificity. The testing of chemokine derivatives or analogues for their ability to bind chemokine receptors using phage display has been described in the treatment and prevention of HIV (U.S. Patent 6,214,540; DeVico et al.).

Another approach for modifying chemokines is phage display. For instance, phage display techniques have been used to detect or identify ligands, inhibitors or promoters of receptor proteins for CXC Chemokine Receptor 3 (CXCR3) ( U.S. Patent 6,140,064, Loetscher et al.), which are characterized by selective binding of one or more chemokines with the ability to induce a cellular response (U.S. Patent 6,184,358, Loetscher et al.). The use of phage display has been described in the labeling and selection of molecules (U.S. Patent 6,180,336, Osbourn et al.), the labeling and subsequent purification of binding molecules for specific antigens (see e.g., WO92/01047), and in the determination of peptide composition for prevention and treatment of HIV infection and immune disorders (U.S. Patent 6,090,388, Wang). (CITE OFFORD).

## B. Exemplary Synthetic Chemokines of the Present Invention

The most preferred synthetic chemokine proteins of the invention, are derivatives of Rantes (alternatively referred to as Nonakines, or "NK"). The more preferred Nonakines of the invention are described in **Table 3**.

**Table 3: Exemplary synthetic Rantes derivatives\***

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J-X<sup>1</sup>X<sup>2</sup>X<sup>3</sup>SSDX<sup>7</sup>X<sup>8</sup>PC CFAX<sup>14</sup>IAAX<sup>17</sup>PLP RAHIKX<sup>26</sup>YFYT SGKCSNPAVV  
FVTX<sup>44</sup>X<sup>45</sup>NX<sup>47</sup>QVC ANPEKKWVRE YINSX<sup>65</sup>X<sup>66</sup>X<sup>67</sup>X<sup>68</sup>-Z

(SEQ ID NO.1)

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\*J is an N-terminal capping group (e.g., one or more additional amino acids, a polymer, a chemical adduct, and the like) that may be present or absent; X<sup>1</sup>, X<sup>2</sup>, X<sup>3</sup>, X<sup>7</sup>, X<sup>8</sup>, X<sup>14</sup>, X<sup>17</sup>, X<sup>26</sup>, X<sup>44</sup>, X<sup>45</sup>, X<sup>47</sup>, X<sup>65</sup>, X<sup>66</sup>, X<sup>67</sup> and X<sup>68</sup> are each individually an amino acid selected from the corresponding position in a wild type Rantes, an amino acid substitution, deletion, or polymer attachment residue; and Z is N-terminal capping group (e.g., one or more additional amino acids, a polymer, a chemical adduct, and the like), or a polymer attachment residue, and may be present or absent.

In reference to **Table 3** and **SEQ ID NO:1**, preferred Nonakines have one or more of the following modifications:

J = N-terminal capping group (e.g., one or more additional amino acids, a polymer, a chemical adduct, and the like) that may be present or absent.

X<sup>1</sup> = Serine, amino acid derivative (e.g., glyoxyl), or N-terminal capping group (e.g., one or more additional amino acids, a polymer, a chemical adduct, and the like) that may be present or absent.

X<sup>2</sup> = Proline, amino acid derivative (e.g., thioproline), or N-terminal capping group (e.g., one or more additional amino acids, a polymer, a chemical adduct, and the like) that may be present or absent. When any one or more of J – X<sup>1</sup> is absent, may include an N-terminal deletion up through the X<sup>1</sup>, where X<sup>2</sup> can be a proline, amino acid derivative (e.g., cyclohexyl glycine), or N-terminal capping group (e.g., one or more additional amino acids, a polymer, a chemical adduct, and the like) that may be present or absent.

X<sup>3</sup> = Tyrosine, amino acid derivative (e.g., cyclohexyl glycine), or N-terminal capping group (e.g., one or more additional amino acids,

a polymer, a chemical adduct, and the like) that may be present or absent. When any one or more of J – X<sup>2</sup> is absent, may include an N-terminal deletion up through X<sup>2</sup>, where X<sup>3</sup> can be a tyrosine, amino acid derivative (e.g., cyclohexyl glycine), or N-terminal capping group (e.g., one or more additional amino acids, a polymer, a chemical adduct, and the like) that may be present or absent.

X<sup>7</sup> = Threonine, amino acid derivative (e.g., N-methyl threonine) that may be present or absent. When any one or more of J – X<sup>6</sup> is absent, may include an N-terminal deletion up through the aspartic acid at position 6, where X<sup>7</sup> can be a threonine, amino acid derivative (e.g., N-methyl threonine), or N-terminal capping group (e.g., one or more additional amino acids, a polymer, a chemical adduct, and the like) that may be present or absent.

X<sup>8</sup> = Threonine, amino acid derivative (e.g., N-methyl threonine), or N-terminal capping group (e.g., one or more additional amino acids, a polymer, a chemical adduct, and the like) that may be present or absent. When any one or more of J – X<sup>7</sup> is absent, may include an N-terminal deletion up through the X<sup>7</sup>, where X<sup>8</sup> can be a threonine, amino acid derivative (e.g., N-methyl threonine), or N-terminal capping group (e.g., one or more additional amino acids, a polymer, a chemical adduct, and the like) that may be present or absent.

X<sup>14</sup> = Tyrosine, alanine, or amino acid derivative (e.g. cyclohexyl glycine).

X<sup>17</sup> = Arginine, alanine, amino acid derivative (e.g. amino-butyric acid (Abu)), or a polymer attachment residue comprising an amino acid or amino acid analog bearing a side chain covalently joinable, or joined to a water-soluble polymer (e.g., side chain bearing protected or unprotected functional group (e.g., aminooxy, levulinic acid, pyruvic acid, amino, thiol, selenol, thioester, selenoester), or side chain attached to a polymer through a covalent bond (e.g., oxime, hydrazone, amide, thioester, selenoester, thioether, selenoester, oxazolidine, thiazolidine) and the like.

X<sup>26</sup> = Glutamic acid, alanine, or amino acid derivative (e.g., Abu).

X<sup>44</sup> = Arginine, alanine, amino acid derivative (e.g., Abu), or a polymer attachment residue as for X<sup>17</sup>.

X<sup>45</sup> = Lysine, alanine, amino acid derivative (e.g., Abu), or a polymer attachment residue as for X<sup>17</sup>.

X<sup>47</sup> = Arginine, alanine, amino acid derivative (e.g., Abu), or a polymer attachment residue as for X<sup>17</sup>.

X<sup>65</sup> = Leucine or amino acid derivative (e.g., leu-amide). When X<sup>66</sup> – Z is absent, may be leucine, amino acid derivative, or Z.

- $X^{66}$  = Glutamic acid, alanine, or amino acid derivative (e.g., glu-amide), and may be present or absent. When  $X^{67} - Z$  is absent, may be glutamic acid, alanine, amino acid derivative, or Z.
- $X^{67}$  = Methionine, serine, alanine, amino acid derivative (e.g., met-amide, norleucine), or a polymer attachment residue as for  $X^{17}$ , and may be present or absent. When  $X^{68} - Z$  is absent, may be methionine, serine, alanine, amino acid derivative, a polymer attachment residue, or Z.
- $X^{68}$  = Serine, alanine, amino acid derivative (e.g., ser-amide, amino-butyric acid (Abu)), or a polymer attachment residue as for  $X^{17}$ , and may be present or absent.
- Z = C-terminal capping group (e.g., one or more additional amino acids, a polymer, a chemical adduct, and the like), or a polymer attachment residue as for  $X^{17}$ , and may be present or absent.

In a preferred embodiment, the synthetic chemokines of the invention have an amino acid sequence of **SEQ ID NO:1** and the biological activity of down-regulating at least one corresponding Rantes receptor. Synthetic chemokines having an amino acid sequence of **SEQ ID NO:1** preferably include at least one modifications as described above at one or more positions selected from the group consisting of J, 1, 2, 3, 4, 7, 8, 14, 17, 26, 44, 45, 47, 66, 67, 68 and Z. Synthetic chemokines having an amino acid sequence of **SEQ ID NO:1** may further include a modification at one or more additional positions as well. More preferably, synthetic chemokines having an amino acid sequence of **SEQ ID NO:1** comprise a modification at one or more of positions 44, 45, 47 in combination with a modification at one or more of positions 26 and 66. Even more preferred are combinations that include a modification at one or more of positions 44, 45, 47 in combination with a modification at one or more of positions 26 and 66, and in combination with a modification at one or more of the positions J, 1, 2 and 3.

In another preferred embodiment, synthetic chemokines having an amino acid sequence of **SEQ ID NO:1** and the biological activity of down-regulating at least one corresponding Rantes receptor comprise a C-terminal truncation involving a deletion of one or more amino acids selected from positions 66, 67 and 68. Synthetic chemokines having an amino acid sequence of **SEQ ID NO:1** and a C-terminal truncation involving a deletion of one or more amino acids selected from positions 66, 67 and 68, also preferably include at least one modification as described above

at one or more positions selected from the group consisting of J, 1, 2, 3, 4, 7, 8, 14, 17, 26, 44, 45, 47 and Z. More preferably, synthetic chemokines having an amino acid sequence of **SEQ ID NO:1** comprise and a C-terminal truncation involving a deletion of one or more amino acids selected from positions 66, 67 and 68, in combination with a modification at one or more of positions J, 1, 2, 3, 44, 45, 47. Even more preferred are combinations that include a C-terminal truncation involving a deletion of one or more amino acids selected from positions 66, 67 and 68, in combination with a modification at one or more of positions J, 1, 2 and 3, in combination with one or more modifications at positions 44, 45, 47. The most preferred synthetic chemokines having an amino acid sequence of **SEQ ID NO:1** and a C-terminal truncation comprise a C-terminal truncation involving the deletion of amino acids C-terminal to the end of the core helix region at position 65, i.e., deletion of the amino acids at positions 66, 67, and 68.

The most preferred Nonakines of the invention are described in **Table 4**, and as described in detail in the Examples.

**Table 4:** Amino acid sequence and modifications with site-specific changes relative to PSC-RANTES depicted in **SEQ ID NO.: 2**, as shown below.

**B<sup>1</sup>X<sup>2</sup>Z<sup>3</sup>**SSDT<sup>7</sup>TPC CFAY<sup>14</sup>IAR<sup>17</sup>PLP RAHIKE<sup>26</sup>YFYT SGKCSNPAVV  
FVTR<sup>44</sup>K<sup>45</sup>NR<sup>47</sup>QVC ANPEKKWVRE YINSLE<sup>66</sup>M<sup>67</sup>S<sup>68</sup> (**SEQ ID NO: 2**)

**B<sup>1</sup>** = Nonanoyl; **X<sup>2</sup>** = Thioproline; **Z<sup>3</sup>** = Cyclohexylglycine

Cmpd.	Backbone modifications relative to SEQ ID NO: 2											
	7	14	17	26	44	45	47	66	67	68	69	70
NK	T	Y	R	E	R	K	R	E	K <sup>p1p</sup>		K <sup>pal</sup>	
NK 1	T	A	A	A	A	A	A	S	M	S	K <sup>p1p</sup>	L
NK 2	T	A	A	E	A	K <sup>p1p</sup>	A	E	M	S	Δ	Δ
NK 3	T	Y	R	E	R	K <sup>p1p</sup>	R	Δ	M	S	Δ	Δ
NK 4	T	Y	R	E	R	K <sup>p1p</sup>	R	Δ	Δ	Δ	Δ	Δ
NK 7	NmeT	A	A	A	A	A	A	S	M	S	K <sup>p1p</sup>	L
NK 8	T	A	A	A	A	K <sup>p1p</sup>	A	Δ	M	S	Δ	Δ
NK 6	T	A	K <sup>p1p</sup>	A	A	A	A	S	M	S	Δ	Δ
Nk 5	NmeT	A	A	A	R	K <sup>p1p</sup>	R	Δ	M	S	Δ	Δ
NK 9	T	Y	R	E	R	K <sup>p1p</sup>	R	Δ	M	K <sup>pal</sup>	Δ	Δ
Nk 11	T	Y	R	E	R	K <sup>peg</sup>	R	Δ	M	S	Δ	Δ

NK 10	T	Y	R	E	R	K <sup>peg</sup>	R	Δ	M	S	Δ	Δ
NK 13	T	Y	R	E	R	K <sup>peg</sup>	R	Δ	Δ	Δ	Δ	Δ

Key: plp = precision length polyamide polymer; PEG = polyethylene glycol; Pal = palmitate derivative; Δ = deletion or absent. All amino acids depicted using single letter code. The bond connecting the lysine and plp are levulinic-aminoxy, while the bond connecting the lysine and PEG are aminoxy-aldehyde.

## II. SYNTHESIS OF THE SYNTHETIC CHEMOKINES OF THE PRESENT INVENTION

The synthetic chemokines of the invention can be prepared by numerous methods, including by recombinant DNA expression (synthesis in part or in total), total chemical synthesis (synthesis in total) and semi-synthesis (synthesis in total) employing a combination of recombinant DNA expression and chemical synthesis. For the C-terminally truncated synthetic chemokines of the invention that are produced in total by recombinant DNA expression, such molecules are generally those that are devoid of non-genetically encodable modifications (e.g., the chemokine polypeptide chain contains one or more amino acids that are not ribosomally installed).

Accordingly, the preferred method for synthesis involves at least one aspect of chemical synthesis, for example, step-wise chain assembly of amino acids (solid phase peptide synthesis), and/or chemical ligation of peptide segments (e.g., native chemical ligation), and/or post chain assembly methods (e.g., attachment of chemical adducts, polymers etc.). Peptide synthesis is preferably based on the "Merrifield"-chemistry stepwise solid phase peptide synthesis protocol developed in the early 1960's, using standard automated peptide synthesizers. The peptide ligation step may employ solid or solution phase ligation strategies. Chemical ligation involves the formation of a selective covalent linkage between a first chemical component and a second chemical component. Unique, mutually reactive, functional groups present on the first and second components can be used to render the ligation reaction chemoselective. For example, the chemical ligation of peptides and polypeptides involves the chemoselective reaction of peptide or polypeptide

segments bearing compatible unique, mutually-reactive, C-terminal and N-terminal amino acid residues.

In one embodiment, all of the amino acid residues of the synthetic bioactive protein may be joined together by a peptide bond (i.e., an amide bond). Alternatively, two amino acid residues (or the C-terminal and N-terminal residues of two polypeptides) may be linked to one another by a non-amide bond (such as a thioester bond, an oxime bond, a thioether bond, a directed disulfide bond, a thiozolidine bond, hydrazone forming ligation, oxazolidine forming ligation, etc.) (Schnölzer, M. and Kent, S.B.H., *Science* (1992) 256:221-225; Rose, K., *J. Amer. Chem. Soc.* (1994) 116:30-33; Englebrechtsen, D.R. *et al.*, *Tetrahedron Lett.* 36:8871-8874; Baca, M. *et al.*, *J. Amer. Chem. Soc.* (1995) 117:1881-1887; Liu, C.F. *et al.*, *J. Amer. Chem. Soc.* (1994) 116:4149-4153; Liu, C.F. *et al.*, *J. Amer. Chem. Soc.* (1996) 118:307-312; Dawson, P.E. *et al.* (1994) *Science* 266:776-779; Gaertner, *et al.*, *Bioconj. Chem.* (1994) 5(4):333-338; Zhang, *et al.*, *Proc. Natl. Acad. Sci.* (1998) 95(16):9184-9189; Tam, *et al.*, WO 95/00846; US Patent No. 5,589,356) or by other methods (Yan, L.Z. and Dawson, P.E., "Synthesis of Peptides and Proteins without Cysteine Residues by Native Chemical Ligation Combined with Desulfurization," *J. Am. Chem. Soc.* 2001, 123, 526-533, herein incorporated by reference; Gieselmann *et al.*, *Org. Lett.* 2001 3(9):1331-1334; Saxon, E. *et al.*, "Traceless" Staudinger Ligation for the Chemoselective Synthesis of Amide Bonds. *Org. Lett.* 2000, 2, 2141-2143. The invention thus permits a variety of peptide bond modifications, surrogates and isosteric replacements to be exploited in the preparation of bioactive proteins.

Where the ligation involves the joining of a polypeptide that possesses an N-terminal cysteine residue, the procedure of native chemical ligation is preferably employed (Dawson, *et al.*, *Science* (1994) 266:776-779; Kent, *et al.*, WO 96/34878; Kent, *et al.*, WO 98/28434)). This methodology has proven a robust methodology for generating a native amide bond at the ligation site. Native chemical ligation involves a chemoselective reaction between a first peptide or polypeptide segment having a C-terminal  $\alpha$ -carboxythioester moiety and a second peptide or polypeptide having an N-terminal cysteine residue. A thiol exchange reaction yields an initial thioester-linked intermediate, which spontaneously rearranges to give a native amide bond at the ligation site while regenerating the cysteine side chain thiol. In many instances,

the sequence of the natural protein will comprise suitably placed cysteine residues such that polypeptide fragments having an N-terminal cysteine residue may be synthesized and used in a native chemical ligation reaction. In other instances, the peptide synthesis can be conducted so as to introduce cysteine residues into a polypeptide for this purpose.

Attachment of polymers to polypeptides is well known, and can be more or less driven by selection of an attachment chemistry relative to the reactive groups present on the chemokine polypeptide chain, or preferably, more site-specific in nature. In general, polymer attachment may be to the N- or C-terminus, side chain of the naturally occurring amino acid, an amino acid derivative that replaces the naturally occurring amino acid, or through another moiety that is attached at the target position suitable for this purpose. Chemistries suitable for attaching polymers to proteins are well known. See for example, WO 02/04014; ROSE; Perspectives in Bioconjugate Chemistry, Claude F. Meares, Ed., American Chemical Society, 1993; Abuchowski, *et al*, *J. Biol. Chem.* (1977) 252(11):3578-3581; Tsutsumi, *et al*, *Jpn. J. Cancer Res.* (1994) 85:9-12; Poly(ethylene glycol) Chemistry and Biological Applications, ACS Symposium Series 680, J.M. Harris and S. Zalipsky, Eds., American Chemical Society, 1997; and Poly(ethylene glycol) Chemistry, Biotechnical and Biomedical Applications, Topics in Applied Chemistry, J.M. Harris, Ed., Plenum Press, New York, NY, 1992).

In a preferred embodiment, the invention provides a method of producing a synthetic chemokine in a substantially purified oligomeric form. This method involves: synthesizing a protein pool containing a synthetic chemokine protein comprising a chemokine polypeptide chain having an N-terminus and a C-terminus, where the chemokine polypeptide chain comprises (i) an amino acid sequence and cysteine pattern corresponding to a wild type chemokine, and (ii) a C-terminal truncation relative to the wild type chemokine; and purifying from said protein pool one or more oligomeric forms of said synthetic chemokine protein so as to produce a synthetic chemokine in a substantially single oligomeric form.

Also provided are methods of producing the bioactive synthetic chemokines of the present invention. The method involves (i) synthesizing an analog of a wild type that comprises a polypeptide chain having an amino acid sequence that is



substantially homologous to the wild type chemokine, where the polypeptide chain comprises a C-terminal truncation and is modified at one or more of its N-terminus, N-loop and C-terminus with a moiety selected from an aliphatic chain, an amino acid derivative, and a polymer; and (ii) screening the chemokine analog for binding to a chemokine receptor of the corresponding wild type chemokine.

Synthesis of the synthetic chemokines of the invention is preferably accomplished by chemical synthesis (i.e., ribosomal-free synthesis), or a combination of biological (i.e., ribosomal synthesis) and chemical synthesis. For chemical synthesis, the synthetic chemokines of the present invention can be made *in toto* by stepwise chain assembly or fragment condensation techniques, such as solid or solution phase peptide synthesis using Fmoc and tBoc approaches, by chemical ligation of peptide segments made *in toto* by chain assembly, or a combination of chain assembly and biological production. Such stepwise chain assembly or fragment condensation and ligation techniques are well known in the art (See, e.g., Kent, S.B.H., *Ann. Rev. Biochem.* (1988) 57:957-989; Dawson *et al.*, *Methods Enzymol.* (1997) 287:34-45; Muir *et al.*, *Methods Enzymol.* (1997) 289:266-298; Wilken *et al.*, *Current Opinion In Biotechnology* (1998) 9:412-426; Ingenito *et al.*, *J. Amer. Chem. Soc.* (1999) 121(49):11369-11374; and Muir *et al.*, *Chemistry & Biology* (1999) 6:R247-R256).

For chemical ligation, a first peptide segment having an N-terminal functional group is ligated to a second peptide segment having a C-terminal functional group that reacts with the N-terminal functional group to form a covalent bond therein between. Depending on the functional groups selected, the ligation reaction generates a product having a native amide bond or a non-native covalent bond at the ligation site. The first or second peptide segment employed for chemical ligation is typically made using stepwise chain assembly or fragment condensation. In particular, when the bioactive synthetic chemokines of the present invention are made by ligation of peptide segments, the segments are made to contain the appropriate pendant chemoselective reactive groups with respect to the intended chemoselective reaction chemistry to be used for ligation. These chemistries include, but are not limited to, native chemical ligation (Dawson, *et al.*, *Science* (1994) 266:776-779; Kent, *et al.*, WO 96/34878), extended general chemical ligation

(Kent, *et al.*, WO 98/28434), oxime-forming chemical ligation (Rose, *et al.*, *J. Amer. Chem. Soc.* (1994) 116:30-33), thioester forming ligation (Schnölzer, *et al.*, *Science* (1992) 256:221-225), thioether forming ligation (Englebretsen, *et al.*, *Tet. Letts.* (1995) 36(48):8871-8874), hydrazone forming ligation (Gaertner, *et al.*, *Bioconj. Chem.* (1994) 5(4):333-338), and thiazolidine forming ligation and oxazolidine forming ligation (Zhang, *et al.*, *Proc. Natl. Acad. Sci.* (1998) 95(16):9184-9189; Tam, *et al.*, WO 95/00846).

Reaction conditions for a given ligation chemistry are selected to maintain the desired interaction of the ligation components. For example, pH and temperature, water-solubility of the peptides and components, ratio of peptides, water content and composition of the individual peptides can be varied to optimize ligation. Addition or exclusion of reagents that solubilize the peptides to different extents may further be used to control the specificity and rate of the desired ligation reaction. Reaction conditions are readily determined by assaying for the desired chemoselective reaction product compared to one or more internal and/or external controls.

A preferred method of chemical synthesis employs native chemical ligation, which is disclosed in Kent *et al.*, WO 96/34878, and a method of preparing proteins chemically modified at the N- and/or C-terminal is disclosed in Offord *et al.*, WO 99/11666, the disclosures of which are incorporated herein by reference. In general, a first peptide containing a C-terminal thioester is reacted with a second peptide with an N-terminal cysteine having an unoxidized sulfhydryl side chain. The unoxidized sulfhydryl side chain of the N-terminal cysteine is condensed with the C-terminal thioester in the presence of a catalytic amount of a thiol, preferably benzyl mercaptan, thiophenol, 2-nitrothiophenol, 2-thiobenzoic acid, 2-thiopyridine, and the like. An intermediate peptide is produced by linking the first and second peptides via a  $\beta$ -aminothioester bond, which rearranges to produce a peptide product comprising the first and second peptides linked by an amide bond.

For making synthetic chemokines containing non-genetically encoded amino acid residues by a combination of chemical and biological production, one peptide segment is made by chemical synthesis while the other is made using recombinant approaches, which segments are then joined using chemical ligation to generate the full-length product. For instance, intein expression systems can be utilized to exploit

the inducible self-cleavage activity of an 'intein' protein-splicing element to generate a C-terminal thioester peptide segment. In particular, the intein undergoes specific self-cleavage in the presence of thiols such as DTT, b-mercaptoethanol or cysteine, which generates a peptide segment bearing a C-terminal thioester. (See, e.g., Muir *et al.*, *Chemistry & Biology* (1999) 6:R247-R256; Chong *et al.*, *Gene* (1997) 192:277-281; Chong *et al.*, *Nucl. Acids Res.* (1998) 26:5109-5115; Evans *et al.*, *Protein Science* (1998) 7:2256-2264; and Cotton *et al.*, *Chemistry & Biology* (1999) 6(9):247-256). This C-terminal thioester bearing peptide segment may then be utilized to ligate a second peptide bearing an N-terminal thioester-reactive functionality, such as a peptide segment having an N-terminal cysteine as employed for native chemical ligation.

Polymers, aliphatic chains, amino acid derivatives and the like can be incorporated during chain assembly, post chain assembly or a combination thereof. For example, for incorporation during chain assembly, the amino acid derivatives and/or amino acids having an aliphatic chain attached thereto are incorporated in the stepwise or fragment condensation, and/or the ligation chain assembly process. These amino acids can be added in a stepwise fashion to the growing peptide chain during peptide synthesis, to assembled peptide segments targeted for ligation, or in some instances the pendant N- or C-terminal modifications can be provided by cleavage from a polymer support, whereby the cleavage product yields the desired hydrophobic aliphatic chain. For post chain assembly, amino acids or derivatives thereof having a reactive functional group are incorporated during chain assembly (in protected or unprotected form) which are then utilized in their unprotected reactive form for attachment of the desired hydrophobic moiety, i.e., in a post-peptide synthesis conjugation reaction. The post chain assembly attachment can be performed on a denatured linear peptide chain, or following folding of the polypeptide chain. In a preferred embodiment, the amino acid derivative is added during peptide synthesis at an amino acid position of interest, whereas the N-, C- and/or N-/C-terminal hydrophobic aliphatic chain is added following peptide synthesis through a conjugation reaction. Any of numerous conjugation chemistries can be utilized (See, e.g., Plaue, S *et al.*, *Biologicals*. (1990) 18(3):147-57; Wade, J.D. *et al.*, *Australas Biotechnol.* (1993) 3(6):332-6; Doscher, M.S., *Methods Enzymol.* (1977) 47:578-617; Hancock, D.C. *et al.*, *Mol Biotechnol.* (1995) 4(1):73-86; Albericio, F. *et al.*, *Methods*

*Enzymol.* (1997) 289:313-36), as well as ligation chemistries, depending on the desired covalent linkage. Folding of the bioactive synthetic chemokines of the present invention can be achieved following standard techniques in the art. See, e.g., WO 99/11655; WO 99/11666; Dawson *et al.*, *Methods Enzymol.* (1997) 287:34-45).

For screening the synthesized chemokine compounds for antagonist activity, the compounds are examined by in vitro or in vivo based assays characterized by direct or indirect binding of the chemokine ligand to its corresponding receptor. Examples of chemokine receptors and their corresponding wild type chemokine include CXCR1 (Fractalkine); XCR1 (SCM-1); CXCR2 (GRO, LIX, MIP-2); CXCR3 (MIG, IP-10); CXCR4 (SDF-1); CXCR5 (BLC); CCR1 (MIP-1 $\alpha$ , RANTES, MCP-3); CCR2 (MCP-1, MCP-3, MCP-5); CCR3 (Eotaxin, RANTES, MIP-1 $\alpha$ ); CCR4 (MDC, TARC); CCR5 (RANTES, MIP-1 $\alpha$ , MIP-1 $\beta$ ); CCR6 (MIP-3 $\alpha$ ); CCR7 (SLC, MIP-3 $\beta$ ); CCR8 (TCA-3); and CCR9 (TECK). In vitro and in vivo assays for these systems are well known, and readily available or can be created de novo. See, e.g., US 5,652,133; US 5,834,419; WO 97/44054; WO 00/04926; and WO 00/0492. For instance, natural, transformed, and/or transgenic cell lines expressing one or more chemokine receptors are typically used to monitor the effect of chemokine-induced chemotaxis or the inhibition of this event when exposed to a bioactive synthetic chemokine of the present invention. Animal models also may be employed, for example, to monitor a response profile in conjunction with treatment with a bioactive synthetic chemokine of the present invention, or to characterize the pharmacokinetic and pharmacodynamic properties of the compounds. To characterize the compounds of the invention as inhibitors of viral infection, envelope-mediated cell fusion assays employing a target cell line and an envelop cell line may be employed for screening bioactive synthetic chemokines of the present invention for their ability to prevent HIV infection. Of course, cell-free viral infection assays may be employed as well for this purpose.

As an example, for assessing antagonism of chemotaxis in general, peripheral blood leukocytes can be employed, such as those isolated from normal donors according to established protocols for purification of monocytes, T lymphocytes and neutrophils. A panel of C, CC, CXXXC and CXC chemokine

receptor-expressing test cells can be constructed and evaluated following exposure to serial dilutions of individual compounds of the invention. Native chemokines can be used as controls. For instance, a panel of cells transfected with expression cassettes encoding various chemokine receptors are suitable for this purposes. For instance, antagonist of chemokines such as RANTES, SDF-1 $\alpha$  or SDF-1 $\beta$  and MIP can be screened using transformants expression CXCR4/Fusion/LESTR, CCR3, CCR5, CXCR4 (such cells are available from various commercial and/or academic sources or can be prepared following standard protocols; see, e.g., Risau, *et al.*, Nature 387:671-674 (1997); Angiololo, *et al.*, *Annals NY Acad. Sci.* (1996) 795:158-167; Friedlander, *et al.*, *Science* (1995) 870:1500-1502). The results can be expressed as the chemotaxis index ("CI") representing the fold increase in the cell migration induced by stimuli versus control medium, and statistical significance determined.

Receptor binding assays also can be performed, for example, to evaluate competitive inhibition versus receptor recycling effects (see, Signoret, N. *et al.*, "Endocytosis and recycling of the HIV coreceptor CCR5," *J Cell Biol.* 2000 151(6):1281-94; Signoret, N. *et al.*, "Analysis of chemokine receptor endocytosis and recycling," *Methods Mol Biol.* 2000;138:197-207; Pelchen-Matthews, A. *et al.*, "Chemokine receptor trafficking and viral replication," *Immunol Rev.* 1999 Apr;168:33-49; Daugherty, B.L. *et al.*, "Radiolabeled chemokine binding assays," *Methods Mol Biol.* 2000;138:129-34; Mack, M. *et al.* "Downmodulation and recycling of chemokine receptors," *Methods Mol Biol.* 2000;138:191-5; all herein incorporated by reference). This approach is well known and typically will employ labeled bioactive synthetic chemokines of the present invention in the presence of increasing concentrations of unlabeled native chemokines following standard protocols. Of course labeling can be on either or both ligands. In this type of assay, the binding data can be analyzed, for example, with a computer program such as LIGAND (P.Munson, Division of Computer Research and Technology, NIH, Bethesda, MD), and subjected to Scatchard plots analysis with both "one site" and "two site" models compared to native leukocytes or the panel of receptor-transfected cells expressing a target chemokine receptor. The rate of competition for binding by unlabeled ligands can then be calculated with the following formula: % inhibition =  $1 - (\text{Binding})$

in the presence of unlabeled chemokine/binding in the presence of medium alone) x 100.

For screening the compounds for their ability to prevent or alleviate viral infection and disease, the compounds can be screened against a panel of cells stably expressing either the appropriate receptor exposed to various viral strains and controls. For instance, U87/CD4 cells expressing CCR3, CCR5, CXCR4 or CXCR4 receptors can be employed for screening infection of M-tropic, T-tropic and dual tropic HIV strains. Inhibition of viral infection can be accessed as a percentage of infection relative to the concentration of chemokine and control concentrations. See., e.g., McKnight, *et al.*, *Virology* (1994) 201:8-18); and Mosier, *et al.*, *Science* (1993) 260:689-692; Simmons, *et al.*, *Science* (1997) 276:276-279; Wu, *et al.*, *J. Exp. Med.* (1997) 185:168-169; and Trkola, *et al.*, *Nature* (1996) 384:184-186). Calcium mobilization assays are another example useful for screening for antagonists of receptor binding, for instance to identify antagonists of native chemokines that are chemotactic for neutrophils and eosinophils (Jose, *et al.*, *J. Exp. Med.* 179:881-887 (1994)). As another example, angiogenic activities of compounds of the invention can be evaluated by the chick chorioallantoic membrane (CAM) assay (Oikawa, *et al.*, *Cancer Lett.* (1991) 59:57-66).

### **III. PHARMACEUTICS, DISEASE STATES AND TREATMENT**

The bioactive synthetic chemokines of the present invention have many uses, including use as research tools, diagnostics and as therapeutics. In particular, the bioactive synthetic chemokines of the present invention have been found to possess valuable pharmacological properties, and have been shown to effectively block the inflammatory effects associated with the corresponding wild type molecules – which are involved in various disorders including asthma, allergic rhinitis, atopic dermatitis, atheroma/atherosclerosis, cancer, organ transplant rejection, and rheumatoid arthritis. Accordingly, they are useful for the treatment of asthma, allergic rhinitis, atopic dermatitis, atheroma/atherosclerosis, organ transplant rejection, and rheumatoid arthritis. For instance, several of the bioactive synthetic chemokines of the present invention such as the RANTES and SDF-1 $\alpha$  or SDF-1 $\beta$  antagonists also have been shown to inhibit HIV-1 infection, and antagonists (e.g., vMIP-II analogues)

can be used for the same purpose. Thus, the RANTES, or SDF-1 $\alpha$  or SDF-1 $\beta$  antagonists and the vMIP-II analogues of the invention can be used for inhibiting HIV-1 in mammals. The potential of the compounds for utility against HIV-1 is determined by the method, described in the following Examples. The potential of the compounds for utility against inflammatory effects is determined by methods well known to those skilled in the art. Moreover, it will be understood that the bioactive synthetic chemokines of the present invention can be utilized alone, or in combination with each other, as well as in combination with other non-chemokine drugs that are synergistic in treating a given disorder.

By way of example, and not by way of limitation, the following are some specific examples of wild type chemokines molecules and their associated biological properties to illustrate the general utility of making the bioactive synthetic chemokines of the present invention. For instance, SCM-1 is a C-Chemokine expressed in spleen. It is substantially related to the CC and CXC-Chemokines, with a primary difference being that it only has the second and fourth of the four cysteines conserved in these proteins (Yoshida *et al.* *FEBS Letters* (1995) 360(2):155-159); Yoshida *et al.* *J. Biol. Chem.* (1998) 273(26):16551-16554). In humans, there are two highly homologous SCM-1 proteins, SCM-1 $\alpha$  and SCM-1 $\beta$ , which differ by two amino acid substitutions. SCM-1 is found to be about 60% identical with lymphotactin, a murine lymphocyte-specific chemokine. SCM-1 and lymphotactin may thus represent the human and murine prototypes of C-Chemokines or Gamma-Chemokines. Both SCM-1 molecules specifically induce migration in murine L1.2 cells engineered to express the orphan receptor, GPR5, which is expressed primarily in placenta, and weakly in spleen and thymus among various human tissues. Accordingly, antagonists of SCM-1 find use in blocking the normal function of GPR4.

As another example, the soluble form of Fractalkine, a 76 amino acid CXXXC-chemokine, is a potent chemoattractant for T-cells and monocytes but not for neutrophils. Fractalkine is increased markedly after stimulation with TNF or IL1. The human receptor for Fractalkine is designated CX3CR1. The receptor mediates both the adhesive and migratory functions of Fractalkine. The human receptor is expressed in neutrophils, monocytes, T-lymphocytes, and several solid organs, including brain. The receptor has been shown to function with CD4 as a coreceptor

for the envelope protein from a primary isolate of HIV-1. A cell-cell fusion assay demonstrates that Fractalkine potently and specifically inhibits fusion. (See, e.g., Bazan *et al* *Nature* (1997) 385(6617):640-644; Combadiere *et al.* *J. Biol. Chem.* (1998) 273(37):23799-23804; Rossi *et al.* *Genomics* (1998) 47(2):163-170; and Faure *et al.* *Science* (2000) 287:2274-2277). It is therefore apparent that antagonists of Fractalkine can find use in the treatment of various arthritic disorders involving the TNF or IL1 pathway, such as arthritis, as well as finding use as a blocker of HIV infection.

Eotaxin is an additional example. This protein is 74 amino acids in length, and is classified as a CC-Chemokine due to its characteristic cysteine pattern. It has been found in the bronchoalveolar lavage of guinea pigs used as a model of allergic inflammation, and implicated in asthma-related disorders. Eotaxin induces substantial eosinophil accumulation at a 1-2 pM dose in the skin without significantly affecting the accumulation of neutrophils. Eotaxin is a potent stimulator of both guinea pig and human eosinophils in vitro. The factor appears to share a binding site with RANTES on guinea pig eosinophils. Eotaxin induces a calcium flux response in normal human eosinophils, but not in neutrophils or monocytes. The response cannot be desensitized by pretreatment of eosinophils with other CC-Chemokines. In basophils Eotaxin induces higher levels of chemotactic response than RANTES, but it only has a marginal effect on either histamine release or leukotriene C4 generation. It also may play a role in chemotaxis of B-cell lymphoma cells. The primary receptor for Eotaxin is CCR3. (See, e.g., Bartels *et al.*, *Biochem. Biophys. Res. Comm.* (1996) 225(3):1045-51; Jose *et al.*, *J. Exp. Med.* (1994) 179:881-887; Ponath *et al.*, *J. Clin. Investigation* (1996) 97(3):604-612; Ponath *et al.*, *J. Exp. Med.* (1996) 183(6):2437-2448; Yamada *et al.*, *Biochem. Biophys. Res. Comm.* (1997) 231(2):365-368). Accordingly, antagonists of Eotaxin can be used as potent modulators of asthma and other eosinophil related allergic disorders.

RANTES is another example of a target chemokine for which antagonists are of particular interest. It is a CC-Chemokine involved in many disorders ranging from inflammation, organ rejection to HIV infection. The synthesis of RANTES is induced by TNF-alpha and IL1-alpha, but not by TGF-beta, IFN-gamma and IL6. RANTES is produced by circulating T-cells and T-cell clones in culture but not by any T-cell lines



tested so far. The expression of RANTES is inhibited following stimulation of T-lymphocytes. RANTES is chemotactic for T-cells, human eosinophils and basophils and plays an active role in recruiting leukocytes into inflammatory sites. RANTES also activates eosinophils to release, for example, eosinophilic cationic protein. It changes the density of eosinophils and makes them hypodense, which is thought to represent a state of generalized cell activation and is associated most often with diseases such as asthma and allergic rhinitis. RANTES also is a potent eosinophil-specific activator of oxidative metabolism. RANTES increases the adherence of monocytes to endothelial cells. It selectively supports the migration of monocytes and T-lymphocytes expressing the cell surface markers CD4 and UCHL1. These cells are thought to be pre-stimulated helper T-cells with memory T-cell functions. RANTES activates human basophils from some select basophil donors and causes the release of histamines. On the other hand RANTES can also inhibit the release of histamines from basophils induced by several cytokines including one of the most potent histamine inducers, MCAF.

RANTES has been shown recently to exhibit biological activities other than Chemotaxis. It can induce the proliferation and activation of killer cells known as CHAK (C-C-Chemokine-activated killer), which are similar to cells activated by IL2. RANTES is expressed by human synovial fibroblasts and may participate in the ongoing inflammatory process in rheumatoid arthritis. High affinity receptors for RANTES (approximately 700 binding sites/cell;  $K_d = 700$  pM) have been identified on the human monocytic leukemia cell line THP-1, which responds to RANTES in chemotaxis and calcium mobilization assays. The chemotactic response of THP-1 cells to RANTES is markedly inhibited by pre-incubation with MCAF (monocyte chemotactic and activating factor) or MIP-1- $\alpha$  (macrophage inflammatory protein). Binding of RANTES to monocytic cells is competed for by MCAF and MIP-1- $\alpha$ . Receptors for RANTES are CCR1, CCR3 and CCR5. The clinical use and significance of antagonists of RANTES is multifold. For instance, antibodies to natural RANTES can dramatically inhibit the cellular infiltration associated with experimental mesangioproliferative nephritis. In addition, natural RANTES appears to be expressed highly in human renal allografts undergoing cellular rejection related to transplant rejection of the kidney (Pattison *et al.*, *Lancet* (1994) 343(8891): 209-11 (1994). Chemically modified forms of RANTES (Aminooxypentane-RANTES or

AOP-RANTES; and n-nonanoyl-RANTES or NNY-RANTES) have been shown to act as an antagonist for the CCR-5 receptor of chemokines and to have the ability to inhibit HIV-1 infection. Accordingly, the antagonist N-, C- and N-/C-terminal modified analogs of RANTES according to present invention are useful as an anti-inflammatory agent in the treatment of diseases such as asthma, allergic rhinitis, atopic dermatitis, organ transplant, atheroma/atherosclerosis and rheumatoid arthritis.

Antagonists of the chemokines SDF-1 $\alpha$  and  $\beta$  are additional examples, which belong to the CXC class of chemokines. SDF-1 $\beta$  differs by having four additional amino acids at the C-terminus. These chemokines are more than 92% identical to their non-human counterparts. SDF-1 is expressed ubiquitously with the exception of blood cells. SDF-1 acts on lymphocytes and monocytes, but not neutrophils in vitro and is a highly potent chemoattractant for mononuclear cells in vivo. It also induces intracellular actin polymerization in lymphocytes. SDF-1 acts both in vitro and in vivo as a chemoattractant for human hematopoietic progenitor cells, giving rise to mixed types of progenitors, and more primitive types. SDF-1 also appears to be involved in ventricular septum formation. Chemotaxis of CD34+ cells is increased in response to a combination of SDF-1 and IL-3. SDF has been shown also to induce a transient elevation of cytoplasmic calcium in these cells. A primary receptor for SDF-1 is CXCR4, which also functions as a major T-lymphocyte coreceptor for HIV1. See, e.g., Aiuti *et al.*, *J. Exp. Med.* (1997) 185(1):111-120 (1997); Bleul *et al.*, *J. Exp. Med.* (1996) 184(3):1101-1109 (1996); Bleul *et al.*, *Nature* (1996) 382(6594):829-833; D'Apuzzo *et al.* *European J. Immunol.* (1997) 27(7):1788-1793; Nagasawa *et al.*, *Nature* (1996) 382:635-638; Oberlin *et al.*, *Nature* (1996) 382(6594):833-835. So for instance, the SDF-1 $\alpha$  or SDF-1 $\beta$  antagonists of the present invention are useful as an anti-inflammatory agent in the treatment of diseases such as asthma, allergic rhinitis, atopic dermatitis, atheroma / atherosclerosis and rheumatoid arthritis. Moreover, the SDF-1 $\alpha$  or SDF-1 $\beta$  antagonists of the invention can be used alone or in combination with other compounds, such as the RANTES antagonist analogs of the invention, for blocking the effects of SDF-1, RANTES, MIP-1 $\alpha$ , and/or MIP-1 $\beta$  in mammals with respect to the recruitment and/or activation of pro-inflammatory cells, or treating or blocking HIV-1 infection.

Accordingly, another aspect of the invention relates to pharmaceutical compositions and methods of treating a mammal in need thereof by administering therapeutically effective amounts of compounds comprising one or more chemokine(s) of the present invention, or pharmaceutically acceptable salts thereof. By "pharmaceutically acceptable salt" is intended to mean a salt that retains the biological effectiveness and properties of the polypeptides of the invention and which are not biologically or otherwise undesirable. Salts may be derived from acids or bases. Acid addition salts are derived from inorganic acids, such as hydrochloric acid, hydrobromic acid, sulfuric acid (giving the sulfate and bisulfate salts), nitric acid, phosphoric acid and the like, and organic acids such as acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, malic acid, malonic acid, succinic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, salicylic acid, p-toluenesulfonic acid, and the like. Base addition salts may be derived from inorganic bases, and include sodium, potassium, lithium, ammonium, calcium, magnesium salts, and the like. Salts derived from organic bases include those formed from primary, secondary and tertiary amines, substituted amines including naturally-occurring substituted amines, and cyclic amines, including isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, ethanolamine, 2-dimethylaminoethanol, tromethamine, lysine, arginine, histidine, caffeine, procaine, hydrabamine, choline, betaine, ethylenediamine, glucosamine, N-alkylglucamines, theobromine, purines, piperazine, piperidine, N-ethylpiperidine, and the like. Preferred organic bases are isopropylamine, diethylamine, ethanolamine, piperidine, tromethamine, and choline.

The term "treatment" as used herein covers any treatment of a disease in a mammal, particularly a human, and includes: (i) preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it; (ii) inhibiting the disease, i.e. arresting its development; or (iii) relieving the disease, i.e. causing regression of the disease.

By the term "a disease state in mammals that is prevented or alleviated by treatment with a bioactive synthetic chemokine of the present invention" as used herein is intended to cover all disease states which are generally acknowledged in

the art to be usefully treated with bioactive synthetic chemokines of the present invention in general, and those disease states which have been found to be usefully prevented or alleviated by treatment with the specific compounds of the invention. These include, by way of illustration and not limitation, asthma, allergic rhinitis, atopic dermatitis, viral diseases, atheroma/atherosclerosis, rheumatoid arthritis and organ transplant rejection.

As used herein, the term "therapeutically effective amount" refers to that amount of a bioactive synthetic chemokine of the present invention which, when administered to a mammal in need thereof, is sufficient to effect treatment (as defined above), for example, as an anti-inflammatory agent, anti-asthmatic agent, an immunosuppressive agent, or anti-autoimmune disease agent to inhibit viral infection in mammals. The amount that constitutes a "therapeutically effective amount" will vary depending on the chemokine derivative, the condition or disease and its severity, and the mammal to be treated, its weight, age, etc., but may be determined routinely by one of ordinary skill in the art with regard to contemporary knowledge and to this disclosure. As used herein, the term "q.s." means adding a quantity sufficient to achieve a stated function, e.g., to bring a solution to a desired volume (e.g., 100 mL).

The chemokines of this invention and their pharmaceutically acceptable salts, i.e., the active ingredient, are administered at a therapeutically effective dosage, i.e., that amount which, when administered to a mammal in need thereof, is sufficient to effect treatment, as described above. Administration of the bioactive synthetic chemokines of the present invention described herein can be via any of the accepted modes of administration for agents that serve similar utilities. As used herein, the terms "bioactive synthetic chemokines of the present invention", "[pharmaceutically acceptable salts of the] polypeptides of the invention" and "active ingredient" are used interchangeably.

The level of the bioactive synthetic chemokines of the present invention present in a formulation can vary within the full range employed by those skilled in the art, e.g., from about 0.01 percent weight (%w) to about 99.99%w of the bioactive synthetic chemokine of the present invention based on the total formulation and about 0.01%w to 99.99%w excipient. More typically, the bioactive synthetic

chemokines of the present invention will be present at a level of about 0.5%w to about 80%w.

While human dosage levels have yet to be optimized for the bioactive synthetic chemokines of the present invention, generally, a daily dose is from about 0.05 to 25 mg per kilogram body weight per day, and most preferably about 0.01 to 10 mg per kilogram body weight per day. Thus, for administration to a 70 kg person, the dosage range would be about 0.07 mg to 3.5 g per day, preferably about 3.5mg to 1.75 g per day, and most preferably about 0.7 mg to 0.7 g per day. The amount of antagonist administered will, of course, be dependent on the subject and the disease state targeted for prevention or alleviation, the nature or severity of the affliction, the manner and schedule of administration and the judgment of the prescribing physician. Such use optimization is well within the ambit of those of ordinary skill in the art.

Administration can be via any accepted systemic or local route, for example, via parenteral, oral (particularly for infant formulations), intravenous, nasal, bronchial inhalation (i.e., aerosol formulation), transdermal or topical routes, in the form of solid, semi-solid or liquid or aerosol dosage forms, such as, for example, tablets, pills, capsules, powders, liquids, solutions, emulsion, injectables, suspensions, suppositories, aerosols or the like. The bioactive synthetic chemokines of the present invention can also be administered in sustained or controlled release dosage forms, including depot injections, osmotic pumps, pills, transdermal (including electrotransport) patches, and the like, for the prolonged administration of the polypeptide at a predetermined rate, preferably in unit dosage forms suitable for single administration of precise dosages. The compositions will include a conventional pharmaceutical carrier or excipient and a bioactive synthetic chemokine of the present invention and, in addition, may include other medicinal agents, pharmaceutical agents, carriers, adjuvants, etc. Carriers can be selected from the various oils, including those of petroleum, animal, vegetable or synthetic origin, for example, peanut oil, soybean oil, mineral oil, sesame oil, and the like. Water, saline, aqueous dextrose, and glycols are preferred liquid carriers, particularly for injectable solutions. Suitable pharmaceutical carriers include starch, cellulose, talc, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium stearate,

sodium stearate, glycerol monostearate, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol, and the like. Other suitable pharmaceutical carriers and their formulations are described in "Remington's Pharmaceutical Sciences" by E. W. Martin.

If desired, the pharmaceutical composition to be administered may also contain minor amounts of non-toxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like, such as for example, sodium acetate, sorbitan monolaurate, triethanolamine oleate, etc.

Although more of the active ingredient may be required, oral administration can be used to deliver the bioactive synthetic chemokines of the present invention using a convenient daily dosage regimen, which can be adjusted according to the degree of prevention desired or in the alleviation of the affliction. For such oral administration, a pharmaceutically acceptable, non-toxic composition is formed by the incorporation of any of the normally employed excipients, such as, for example, pharmaceutical grades of mannitol, lactose, starch, povidone, magnesium stearate, sodium saccharine, talcum, cellulose, croscarmellose sodium, glucose, gelatin, sucrose, magnesium carbonate, and the like. Such compositions take the form of solutions, suspensions, dispersible tablets, pills, capsules, powders, sustained release formulations and the like. Oral formulations are particularly suited for treatment of gastrointestinal disorders. Oral bioavailability for general systemic purposes can be adjusted by utilizing excipients that improve uptake to systemic circulation, such as formulation comprising acetylated amino acids. See, e.g., US 5,935,601 and US 5,629,020.

The compositions may take the form of a capsule, pill or tablet and thus the composition will contain, along with the active ingredient, a diluent such as lactose, sucrose, dicalcium phosphate, and the like; a disintegrant such as croscarmellose sodium, starch or derivatives thereof; a lubricant such as magnesium stearate and the like; and a binder such as a starch, polyvinylpyrrolidone, gum acacia, gelatin, cellulose and derivatives thereof, and the like.

Liquid pharmaceutically administrable compositions can, for example, be prepared by dissolving, dispersing, etc. a bioactive synthetic chemokine of the present invention (about 0.5% to about 20%) and optional pharmaceutical adjuvants

in a carrier, such as, for example, water, saline, aqueous dextrose, glycerol, glycols, ethanol, preservatives and the like, to thereby form a solution or suspension. If desired, the pharmaceutical composition to be administered may also contain minor amounts of nontoxic auxiliary substances such as wetting agents, suspending agents, emulsifying agents, or solubilizing agents, pH buffering agents and the like, for example, sodium acetate, sodium citrate, cyclodextrine derivatives, polyoxyethylene, sorbitan monolaurate or stearate, etc. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania. The composition or formulation to be administered will, in any event, contain a quantity of the active ingredient in an amount effective to prevent or alleviate the symptoms of the subject being treated. For oral administration to infants, a liquid formulation (such as a syrup or suspension) is preferred.

For a solid dosage form containing liquid, the solution or suspension, in for example propylene carbonate, vegetable oils or triglycerides, is preferably encapsulated in a gelatin capsule. For a liquid dosage form, the solution, e.g. in a polyethylene glycol, may be diluted with a sufficient quantity of a pharmaceutically acceptable liquid carrier, e.g. water, to be easily measured for administration.

Alternatively, liquid or semi-solid oral formulations may be prepared by dissolving or dispersing the active ingredient in vegetable oils, glycols, triglycerides, propylene glycol esters (e.g. propylene carbonate) and the like, and encapsulating these solutions or suspensions in hard or soft gelatin capsule shells.

In applying the bioactive synthetic chemokines of the present invention to treatment of the above conditions, administration of the active ingredients described herein are preferably administered parenterally. Parenteral administration is generally characterized by injection, either subcutaneously, intramuscularly or intravenously, and can include intradermal or intraperitoneal injections as well as intrasternal injection or infusion techniques. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, as emulsions or in biocompatible polymer-based microspheres (e.g., liposomes, polyethylene glycol derivatives, poly(D,C)lactide and the like). Suitable excipients are, for example, water, saline,

dextrose, glycerol, ethanol or the like. In addition, if desired, the pharmaceutical compositions to be administered may also contain minor amounts of non-toxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents, solubility enhancers, protein carriers and the like, such as for example, sodium acetate, polyoxyethylene, sorbitan monolaurate, triethanolamine oleate, cyclodextrins, serum albumin etc.

The bioactive synthetic chemokines of the present invention can be administered parenterally, for example, by dissolving such molecules in a suitable solvent (such as water or saline) or incorporation in a liposomal formulation followed, by dispersal into an acceptable infusion fluid. A typical daily dose of a polypeptide of the invention can be administered by one infusion, or by a series of infusions spaced over periodic intervals. For parenteral administration there are especially suitable aqueous solutions of an active ingredient in water-soluble form, for example in the form of a water-soluble salt, or aqueous injection suspensions that contain viscosity-increasing substances, for example sodium carboxymethylcellulose, sorbitol and/or dextran, and, if desired, stabilizers. The active ingredient, optionally together with excipients, can also be in the form of a lyophilisate and can be made into a solution prior to parenteral administration by the addition of suitable solvents.

A more recently devised approach for parenteral administration employs the implantation of a slow-release or sustained-release system, such that a constant level of dosage is maintained. See, e.g., US 3,710,795, US 5,714,166 and US 5,041,292, which are hereby incorporated by reference.

The percentage of the active ingredient contained in such parental compositions is highly dependent on the specific nature thereof, as well as the activity of the polypeptide and the needs of the subject. However, percentages of active ingredient of 0.01% to 10% in solution are employable, and will be higher if the composition is a solid which will be subsequently diluted to the above percentages. Preferably the composition will comprise 0.02-8% of the active ingredient in solution.

Another method of administering the bioactive synthetic chemokines of the present invention utilizes both a bolus injection and a continuous infusion. This is a particularly preferred method when the therapeutic treatment is for the prevention of HIV-1 infection.



Aerosol administration is an effective means for delivering the bioactive synthetic chemokines of the present invention directly to the respiratory tract. Some of the advantages of this method are: 1) it circumvents the effects of enzymatic degradation, poor absorption from the gastrointestinal tract, or loss of the therapeutic agent due to the hepatic first-pass effect; 2) it administers active ingredients which would otherwise fail to reach their target sites in the respiratory tract due to their molecular size, charge or affinity to extra-pulmonary sites; 3) it provides for fast absorption into the body via the alveoli of the lungs; and 4) it avoids exposing other organ systems to the active ingredient, which is important where exposure might cause undesirable side effects. For these reasons, aerosol administration is particularly advantageous for treatment of asthma, local infections of the lung, and other diseases or disease conditions of the lung and respiratory tract.

There are three types of pharmaceutical inhalation devices, nebulizers, inhalers, metered-dose inhalers and dry powder inhalers. Nebulizer devices produce a stream of high velocity air that causes the chemokine derivative (which has been formulated in a liquid form) to spray as a mist which is carried into the patient's respiratory tract. Metered-dose inhalers typically have the formulation packaged with a compressed gas and, upon actuation, discharge a measured amount of the polypeptide by compressed gas, thus affording a reliable method of administering a set amount of agent. Dry powder inhalers administer the polypeptide in the form of a free flowing powder that can be dispersed in the patient's air-stream during breathing by the device. In order to achieve a free flowing powder, the chemokine derivative is formulated with an excipient, such as lactose. A measured amount of the chemokine derivative is stored in a capsule form and is dispensed to the patient with each actuation. All of the above methods can be used for administering the present invention.

Pharmaceutical formulations based on liposomes are also suitable for use with the chemokines of this invention. See, e.g., US 5,631,018, US 5,723,147, and 5,766,627. The benefits of liposomes are believed to be related to favorable changes in tissue distribution and pharmacokinetic parameters that result from liposome entrapment of drugs, and may be applied to the polypeptides of the present

invention by those skilled in the art. Controlled release liposomal liquid pharmaceutical formulations for injection or oral administration can also be used.

For systemic administration via suppository, traditional binders and carriers include, for example, polyethylene glycols or triglycerides, for example PEG 1000 (96%) and PEG 4000 (4%). Such suppositories may be formed from mixtures containing the active ingredient in the range of from about 0.5 w/w% to about 10 w/w%; preferably from about 1 w/w% to about 2 w/w%.

The present invention further provides kits for use as research reagents as well as in the methods of treatment the invention. The kit can comprise, in separate compartments or containers, synthetic chemokine, which can be provided in a desired dosage form for use as a research reagent, or for administration by a desired route (e.g., intravenous, subcutaneous, and the like). The container of the kit can be, for example, a sterile vial or a pre-loaded syringe. Instructions for use of the synthetic chemokine can also be provided in the kit. The components of the kits may be modified commensurate to the disclosure provided above. For example, the kits can comprise one or more additional reagents, such as another therapeutic or drug.

As described above, and further illustrated in the specific Examples that follow, the bioactive synthetic chemokines of the present invention find use as antagonist of the naturally occurring chemokines. In particular, the bioactive synthetic chemokines of the present invention having enhanced potency as an antagonist find use in the analysis and treatment of various disease states, such as asthma, allergic rhinitis, atopic dermatitis, cancer therapy, organ transplant rejection, viral diseases, atheroma/atherosclerosis, rheumatoid arthritis and organ transplant rejection. The bioactive synthetic chemokines of the present invention also can be utilized in designing and screening small molecule antagonist of their cognate receptors. For instance, the structural diversity engineered into the compounds of the invention facilitates a more rational approach in the design, screening and fine tuning of better small molecule compounds for use as medicaments in the treatment of diseases involving the natural activity of chemokine receptors.

**ABBREVIATIONS**

The following abbreviations may be used herein:

Abu = Aminobutyric acid

Acm = acetamidomethyl thiol-protecting group [i.e.,  $-\text{CH}_2\text{NHCOCH}_3$ ]

Aib = aminoisobutyric acid

AoA = aminooxyacetyl

Arg(Tos) = L-arginine(side chain N-toluenesulfonyl-protected)

ART = absolute reticulocyte count

Asp(cHex) = L-aspartic acid(side chain cyclohexyl ester-protected)

AUC = area under the curve

Boc = tert.butoxycarbonyl

Bom = benzyloxymethyl

CD = circular dichroism

CDI = carbonyldiimidazole

CHO = chinese hamster ovary

CL = clearance (mL/hr/kg)

C<sub>max</sub> = maximum concentration

Cys(4MeBzl) = L-cysteine(side chain (4-methyl)benzyl-protected)

Cys(Acm) = L-cysteine(side chain acetamidomethyl [i.e.,  $-\text{CH}_2\text{NHCOCH}_3$ ]-protected)

DBU = Diazabicycloundecane

DCM = dichloromethane

DIC = diisopropylcarbodiimide

DIEA = diisopropylethylamine

DMF = dimethylformamide

Dmg = dimethylglycine

DMSO = dimethylsulfoxide  
Dnp = dinitrophenyl  
DPC = dodecylphosphocholine  
Dpr = L-1,2diaminopropionic acid  
ED50 = effective dose required to reach 50% maximum effect  
EDA = (4,7,10)-trioxatridecane-1,13diamine (also called TTD)  
ELISA = enzyme-linked immunoassay  
ES-MS = electrospray ionization mass spectrometry  
FBS = fetal bovine serum  
Glu(cHex) = L-glutamic acid(side chain cyclohexyl ester-protected)  
HATU = O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate  
His(Dnp) = L-histidine(side chain N1mdinitrophenyl-protected)  
HOBT = N-hydroxybenzotriazole  
HPLC = high pressure liquid chromatography  
IMDM = Iscove's modified Dulbecco's medium  
IPA = isopropanol  
Lev = levulinic acid  
Lys(ClZ) = L-lysine(side chain 2-chlorobenzyloxycarbonyl)-protected  
MBHA = 4-methylbenzhydrylamine  
mcg = microgram  
MRT = mean residence time  
Mtt = 4-methylTrityl  
MTT = thiazolyl blue  
NHS = N-hydroxysuccinimide  
-OCH<sub>2</sub>-Pam-resin = -O-CH<sub>2</sub>-Bz-CH<sub>2</sub>CONHCH<sub>2</sub> (copolystyrene-

divinylbenzene)-resin

Pbo = 4-(CH<sub>3</sub>S(O)-)benzyl

PBS = phosphate buffered saline

RSA = rat serum albumin

SDS = sodium dodecyl sulfate

SDS-PAGE = SDS-polyacrylamide gel electrophoresis

Ser(Bzl) = L-serine(side chain benzyl-protected)

Succ = succinyl

TTD = (4,7,10)-trioxatridecane-1,13diamine (used interchangeable with EDA)

## EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

### Example 1: General Materials and Methods

**Peptide Synthesis:** Peptides for chemokine derivatives were made by solid-phase peptide synthesis as previously described in US Patent Application Serial No. 09/141,833, now US Patent No. 6,168,784. Solid-phase synthesis was performed on a custom-modified 430A peptide synthesizer from Applied Biosystems, using *in situ* neutralization 2-(1H-benzotriazol-1-yl)-1,1,1,3,3-tetramethyluronium hexafluorophosphate activation protocols for stepwise Boc chemistry chain elongation

(Schnolzer, et. al., *Int. J. Peptide Protein Res.* (1992) 40: 180-193). The N-terminal peptide fragments were synthesized on a thioester-generating resin. The resin was split after attachment of the residue preceding the position investigated (elongation from C to N terminus) and the peptide elongated manually. Each synthetic cycle consisted of  $\alpha$ -Boc-removal by a 1 to 2 minute treatment with neat TFA, a 1min DMF flow wash, a 10- to 20-minute coupling time with 1.0 mmol of preactivated Boc-amino acid in the presence of excess DIEA and a second DMF flow wash.  $\alpha$ -Boc-amino acids (1.1 mmol) were preactivated for 3 minutes with 1 mmol HBTU (0.5M in DMF) in the presence of excess DIEA (3mmol). After each manual coupling step, residual free amine was evaluated with the ninhydrin assay (Sarin, et al., *Anal. Biochem.* (1981) 117:147-157). The C-terminal fragment comprising amino acids were synthesized on a standard -O-CH<sub>2</sub>-phenylacetamidomethyl resin. After chain assembly was completed, the peptides were deprotected and cleaved from the resin by treatment with anhydrous HF for 1 hour at 0°C with 5% p-cresol as a scavenger. In all cases, the imidazole side chain DNP protecting groups remained on His residues because the DNP-removal procedure is incompatible with C-terminal thioester groups. However DNP was gradually removed by thiols during the ligation reaction, yielding unprotected His. The side chain of amine group on Lys residues that was used for polymer attachment was protected with Fmoc. After peptide chain assembly, the Fmoc group was removed by 20% piperidine in DMF. Levulinic acid or Isopropylideneaminoxycetic acid (aminooxy-acid whose aminooxy group was protected by acetone) was couple on to the amine group on Lys. After cleavage, both peptides were precipitated with ice cold diethylether, dissolved in aqueous acetonitrile and lyophilized. The peptides were purified by RP-HPLC with a C4-column from Vydacs by using linear gradients of buffer B (acetonitrile/0.081% trifluoroacetic acid) in buffer A (H<sub>2</sub>O/0.1% trifluoroacetic acid) and UV detection at 214nm. Samples were analyzed by electrospray mass spectrometry with a Platform II instrument (Micromass, Manchester, England).

**Native Chemical Ligation:** Unless otherwise noted, peptides were utilized for ligation to generate full length chemokine polypeptide chains using native chemical ligation following standard protocols (Dawson, *et al.*, *Science* (1994) 266:776-779); Wilken, *et. al.*, *Chem. Biol.* (1999) 6:43-51; and Camarero, *et al.*, *Current Protocols in Protein Science* (1999) 18.4.1-18.4.21).

**Folding:** Unless otherwise noted, folding of the polypeptide chains was accomplished in the presence of Cys-SH/(Cys-S)<sub>2</sub> following standard techniques (Wilken *et al.*, *Chem. Biol.* (1999) 6:43-51).

**Chemical Characterization:** The purified materials were characterized by several techniques. For example, C4 reverse HPLC, SEC-MALS (size exclusion-chromatography and multi-angle-light scattering), CD, SDS-PAGE, and ESI-MS. MALDI was used for the pegylated materials. Approximately 50-100 µl of purified pool was injected to C4 reverse phase HPLC column which was equilibrated with 25% buffer A (H<sub>2</sub>O with 0.1% TFA) and 75% buffer B (acetonitrile with 0.08% TFA), the material was eluted with linear gradient from 25-65% A for 20 min. The eluted material was then collected and checked by ESI-MS for its identity. The purity of the material was determined by integration of the main peak and impurity peak of the HPLC chromatography. The aggregation state of the protein was determined by SEC-HPLC-MALS. The MALS and RI (refractive index) detectors from Wyatt Technology Corporation were connected with a HP1100 HPLC which was equipped with Superdex 200 SEC column. The running buffer was 50 mM sodium phosphate, pH 7.4 with 0.5 M NaCl. The molecular weight was calculated by ASTRA software provided by Wyatt Technology Corporation. The aggregation state of the molecule was also investigated by SDS-PAGE gel using NUPAGE 4-2% gel running with MOPS buffer. Far UV CD spectra were obtained utilizing a JASCO 600 instrument. Each curve was the average of 4 scans in the range of 190-260 nm.

## Example 2: RANTES Derivative Peptides and Compounds

The following RANTES derivatives (NKs) were constructed. Peptides used to construct various NK analogs via chemical ligation are set forth below in Table 4. The full-length chemokine polypeptide chain is set forth below in Table 5.

**Table 4: Peptides for NK Analogs**

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G2071:	BXZSSDTTPC	CFAAIAAPLP	RAHIKAYFYT	SGK-thioester
G2088:	CSNPAVV	FVTAANAQVC	ANPEKKWVRE	YINSLMSK <sup>(fmoc)</sup> L-COOH
G2089:	BXZSSDTTPC	CFAAIAAPLP	RAHIKEYFYT	SGK-thioester
G2092:	CSNPAVV	FVTAK <sup>(fmoc)</sup>	NAQVC	ANPEKKWVRE
				YINSLEMS-COOH

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G1832: BXZSSDTPC CFAYIARPLP RAH<sup>(DNP)</sup> IKEYFYT SGK-thioester  
 G2094: CSNPAVV FVTRK<sup>(Fmoc)</sup> NRQVC ANPEKKWVRE YINSLMS-COOH  
 G2095: CSNPAVV FVTRK<sup>(Fmoc)</sup> NRQVC ANPEKKWVRE YINSL-CONH2  
 G2133: BXZSSDT<sup>(Nme)</sup> TPC CFAAIAAPLP RAHIKAYFYT SGK-thioester  
 G2097: CSNPAVV FVTAK<sup>(Fmoc)</sup> NAQVC ANPEKKWVRE YINSLMS-COOH  
 G2136: BXZSSDTPC CFAAIAK<sup>(Fmoc)</sup> PLP RAHIKAYFYT SGK-thioester  
 G2135: CSNPAVV FVTAANAQVC ANPEKKWVRE YINSLSMS-COOH  
 G2142: CSNPAVV FVTRK<sup>(Fmoc)</sup> NRQVC ANPEKKWVRE YINSLMK<sup>(palm)</sup>-COOH  
 G2143: CSNPAVV FVTRK<sup>(aoa-acetone)</sup> NRQVC ANPEKKWVRE YINSLMS-COOH  
 G2163: CSNPAVV FVTRK<sup>(aoa-acetone)</sup> NRQVC ANPEKKWVRE YINSL-CONH2

**B<sup>1</sup>** = Nonanoyl; **X<sup>2</sup>** = Thioproline; **Z<sup>3</sup>** = Cyclohexylglycine

Table 5: Amino acid sequence and modifications with site-specific changes relative to PSC-RANTES depicted in **SEQ ID NO.: 2**, as shown below.

**B<sup>1</sup>X<sup>2</sup>Z<sup>3</sup>**SSDT<sup>7</sup>TPC CFAY<sup>14</sup>IAR<sup>17</sup>PLP RAHIKE<sup>26</sup>YFYT SGKCSNPAVV  
 FVTR<sup>44</sup>K<sup>45</sup>NR<sup>47</sup>QVC ANPEKKWVRE YINSL<sup>66</sup>M<sup>67</sup>S<sup>68</sup> (**SEQ ID NO.: 2**)

**B<sup>1</sup>** = Nonanoyl; **X<sup>2</sup>** = Thioproline; **Z<sup>3</sup>** = Cyclohexylglycine

NK analog	7	14	17	26	44	45	47	66	67	68	69	70	N-terminal peptide	C-terminal peptide
NK 1	T	A	A	A	A	A	A	S	M	S	K <sup>p1p</sup>	L	2071	2088
NK 2	T	A	A	E	A	K <sup>p1p</sup>	A	E	M	S	Δ	Δ	2089	2092
NK 3	T	Y	R	E	R	K <sup>p1p</sup>	R	Δ	M	S	Δ	Δ	1832	2094
NK 4	T	Y	R	E	R	K <sup>p1p</sup>	R	Δ	Δ	Δ	Δ	Δ	1832	2095
NK 7	N <sup>me</sup> T	A	A	A	A	A	A	s	M	S	K <sup>p1p</sup>	L	2133	2088
NK 8	T	A	A	A	A	K <sup>p1p</sup>	A	Δ	M	S	Δ	Δ	2071	2097
NK 6	T	A	K <sup>p1p</sup>	A	A	A	A	s	M	S	Δ	Δ	2136	2135
Nk 5	N <sup>me</sup> T	A	A	A	R	K <sup>p1p</sup>	R	Δ	M	S	Δ	Δ	2133	2094
NK 9	T	Y	R	E	R	K <sup>p1p</sup>	R	Δ	M	K <sup>palm</sup>	Δ	Δ	1832	2142
Nk 11	T	Y	R	E	R	K <sup>peg</sup>	R	Δ	M	S	Δ	Δ	1832	2143
NK 10	T	Y	R	E	R	K <sup>peg</sup>	R	Δ	M	S	Δ	Δ	1832	2143
NK 13	T	Y	R	E	R	K <sup>peg</sup>	R	Δ	Δ	Δ	Δ	Δ	1832	2163



Key: plp = precision length polyamide polymer; PEG = polyethylene glycol; Pal = palmitate derivative; Δ = deletion or absent. All amino acids depicted using single letter code. The bond connecting the lysine and plp are levulinic-aminoxy, while the bond connecting the lysine and PEG are aminoxy-aldehyde.

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### Example 3: Synthesis of the RANTES Derivative Compounds

Synthesis of the NK analogs of Table 5 were constructed as follows.

**Peptide Ligation:** Analogs NK1, NK 2, NK 3, NK 4, NK 7, NK 8, NK 6, NK 5, NK 9, NK 10, NK 11, NK 12 were ligated in the peptide combination listed in Table 4. For peptide ligation a 1.5:1 (peptide 1 : peptide 2 with polymer) molar ratio was used for the peptide ligation. The components were dissolved in freshly prepared 6 M guanidine-HCl/200 mM phosphate, pH 7.0 containing 5 mg/mL L-methionine\* at a concentration of 1 mM. The G2088-PLP peptide was added first, then followed by the G2071 peptide. . Optionally, the solution may be sonicated to aid in dissolving the peptide-PLP and peptide.

Thiophenol is added to the reaction for a concentration of 0.5% under a properly ventilated hood due to the notorious odor of thiophenol. The reaction was gently stirred overnight at room temperature. The progress of the reaction was checked by analytical HPLC with a protein C4 column using a gradient of 5-65% buffer B over 30 min. The peaks were collected and the product peak was identified by mass spec.

After stirring for overnight, one reaction volume of betamercaptoethanol and three reaction volumes of 6 M guanidine-HCl/100 mM tris, pH 8.5 was added to the mixture and stirred for 10 min. The pH of the reaction was then adjusted to 4.0 – 4.5 with glacial acetic acid. TCEP was added to the reaction at 0.25 times the combined weight of peptides and dissolved and stirred for 20 minutes.

The solution was then loaded onto a C4 HPLC column equilibrated with 20% buffer B. The flow through and washes were collected and treated with 10% bleach. For a 5 x 25 cm Vydac C4 column the following parameters were used: Flow Rate: 50 mL/min, Fraction Size: 12.5 mL. Proteins were eluted with 25-45% buffer B over 80 minutes. The fractions were then analyzed by ES-MS and the appropriate

fractions were pooled. The pooled solution was then lyophilized for the next folding reaction.

**Polymer-modification:** To obtain the polymer-modified chemokine, polymer was added either before the protein backbone assembly or after the backbone assembly. Propionyl aldehyde functionalized monomethoxy polyethylene glycol of 5 kD and 20 kD were used for NK 10 and NK 11, respectively. The branched PLP used to modify the other NK analogs was GP 41 or GP43 as described in WO02/19963, herein incorporated by reference.

**a. Polymer attachment protocol #1:** Polymer attachment via oximation between the polymer and peptide bearing the levulinyl group for analogs NK 1, NK 2, NK 3, NK 4, NK 7, NK 8, NK 6, NK 5, NK 9 was as follows. A 1:1.5 (peptide with levulinyl group:bPLP) molar ratio was used for oxime ligation. The components were dissolved in 50% ACN in H<sub>2</sub>O for a PLP concentration of 60 mg/ml. No TFA was added to the 50% CAN/H<sub>2</sub>O solution. The presence of TFA has been demonstrated to decrease the yield of the PLP-peptide product. The solution may also be sonicated to aid in dissolving the PLP and peptide.

Once the peptides were dissolved, the reaction was stirred *gently* overnight at 40 °C. The progress of the reaction was then checked by analytical HPLC with a protein C4 column using a 5-65% B gradient over 30 minutes at 1 ml/min, 40 °C utilizing the gradient (#NEW5-65.M) listed below. The peaks were collected and the product peak was identified by mass spec.

The reaction was then diluted with 2 to 2.5 times of the reaction volume with buffer A to bring the ACN content to below 20%. The resulting solution was loaded onto a C4 HPLC column equilibrated with 20% buffer B. For a 5 x 25 cm Vydac C4 column the following gradient was used: Flow Rate: 50 mL/min, Fraction Size: 12.5 mL. Fractions were collected once the absorbance at 214 nm began to rise. The first peak collected was the unreacted PLP followed by the product and finally, the unreacted peptide.

The fractions were analyzed ES-MS and the appropriate fractions were pooled. These pools were then lyophilized for the next ligation reaction. A typical PLP-peptide yield based on the limiting peptide weight is ~45% after purification.

**b. Polymer attachment protocol #1:** The backbone of NK 10, 11, 12, and 13 proteins was first assembled according to native chemical ligation and folded as above before the acetone protecting group moiety on AoA-Lys residue was taken off and polyethylene glycol (PEG)-aldehyde was attached. Folded protein was then purified by C4 reverse phase column using a buffer of H<sub>2</sub>O-ACN containing 0.1% TFA with a linear gradient of 25-45% over 80 minutes. Fractions were checked by ES-MS and those containing the targeted material were pooled together and lyophilized to dryness.

The dry powder was dissolved into freshly prepared 70% ACN in H<sub>2</sub>O containing 0.1% TFA at 10-15 mg/ml. Methoxyamine HCL was dissolved in the same buffer as the protein at 2 M. Equal volume of protein and methoxyamine solution was mixed and stirred at room temperature for one hour. The acetone moiety on the aminoxy end was then removed and the completion of the reaction was confirmed by ESI-MS. The protein solution was purified by C4 reverse phase column using buffer H<sub>2</sub>O and acetonitrile with 0.1% TFA. A step gradient was used to elute the protein and the pool was lyophilized to dryness.

The dry powder was dissolved at 10 mg/ml in freshly prepared 50% acetonitril-H<sub>2</sub>O buffer containing 0.1% TFA. PEG5Kd-aldehyde and PEG20Kd-aldehyde were dissolved in the same buffer at 20 mg/ml. The peptide solution was mixed with the PEG solution with a 0.5 fold excess of PEG relative to protein. The reaction was stirred at room temperature for 1h. The protein solution was loaded onto a Superdex 200 column which was equilibrated with 50 mM sodium phosphate, pH 7.4 containing 0.5 M NaCl. The protein and PEG was then separated by isocratic gradient. Fractions containing the pegylated protein were pooled together and stored under -80°C.

**Folding:** The following protocols were used for folding.

**a. Folding Protocol #1:** A first round of chemokine analogs were folded by the following protocol. For example, the molecular weight of NK 1 before and after folding were as follows: Unfolded G2071-G2088-PLP = 23,777 Da; Folded G2071-G2088-PLP = 23,773 Da.

An amount of folding buffer (2 M guanidine-HCl/100 mM tris, pH 8.5) required to perform a 0.5 mg/ml G2071-G2088-PLP folding reaction was measured for the protocol. Prior to starting the folding reaction, L-cysteine and L-cystine dihydrochloride was dissolved in the required amount of folding buffer at the following concentrations: 8 mM L-cysteine; 1 mM L-cystine dihydrochloride. The L-cysteine and L-cystine dihydrochloride in folding buffer was freshly prepared for the folding reaction.

While gently stirring, required amount of folding buffer was added to the unfolded G2071-G2088-bPLP. The pH of the reaction was then adjusted to 8.2 - 8.5 with 6 N HCl or 6 N NaOH and stirred for for 90 minutes at room temperature. Typically, trial reactions should be performed prior to performing the bulk folding reaction to determine the appropriate length of time required for the reaction. Extended reaction times have shown evidence of increased aggregation for aggregating analogs such as the base Nonakine (NK).

The reaction was then quenched by adjusting the pH to 4.0 – 4.5 with glacial acetic acid. The purification step should be performed immediately following the quenching of the folding reaction.

The resultant solution was loaded onto a C4 HPLC column equilibrated with 20% buffer B. For a 5 x 25 cm Vydac C4 column the following gradient was used: Flow Rate: 50 mL/min, Fraction Size: 12.5 mL. Fractions were collected once the absorbance at 214 nm begins to rise. The first peak was the folded G2071-G2088-PLP product followed by the misfolded or aggregated G2071-G2088-PLP peak.

Due to the small mass difference between folded and unfolded protein, pooling the fractions requires analysis of mini-pools by analytical HPLC with a protein C4 column using the method #NEW5-65.M as described above. An appropriate pool can be determined by comparing the % purities of each mini-pool to determine which one meets the minimum acceptable purity level. The pool can then be lyophilized for storage or buffer exchanged into an appropriate formulation buffer. A typical folding yield is ~40% after purification.

**b. Folding protocol #2:** The following folding protocol was employed for the remaining NK analogs, unless otherwise specified. For ligated full-length linear polypeptide, the lyophilized powder was dissolved in 7.5 M guanidinium HCl solution

at 0.2- 2 mg/ml. After adding Cysteine and cysteine, the concentrations were 1 and 0.1 mM respectively. Tris buffer was added to a final concentration of 0.1 M at pH 8.5. The protein solution was dialyzed against folding buffer containing 0.6 M guanidinium chloride, 100 mM Tris buffer (pH 8.5), 1 mM cysteine, 0.1 mM cystine for 16-20 hr at 4°C.

The folded protein solution was concentrated up to 8-20 mg/ml by either stirred cell or any similar device with molecular weight cut off at 3500. The concentrated protein solution was loaded onto a Superdex 200 column (26X60 inch) (Amersham Bioscience, MA) which had been equilibrated with 50 mM sodium phosphate, at pH 7.4 containing 0.5 M sodium chloride. The targeted dimer or monomer products were separated with aggregate with isocratic gradient using the equilibration buffer. Fractions were checked by reverse phase HPLC using a C4 column with acetonitrile buffer. Fractions were also check by an analytical Superdex 200 column which ran the same buffer as preparative separation. Fractions containing the targeted material were pooled together and frozen by liquid nitrogen and stored under -80oC.

#### **Example 4: Receptor Signaling by Calcium Flux**

##### **CCR 5 Ca++ flux single transfectant assays**

The NK test articles (labeled CCR#1 through CCR#15) were tested for agonist activity at ten (10) concentrations in duplicate in a cell-based CCR5 Calcium flux assay. Data presented for kinetics studies represent the three highest tested concentrations in addition to the lowest tested concentration for each compound. Concentration response curves were plotted for each test article at maximal RFU. Rank order of potency is presented (in Table 1) which compares each compound at 10 µM expressed as a Fold difference over the diluent control.

Buffer controls for test compounds CCR#1 and CCR#7 were subtracted from the compound values as it was observed the lower pH (6.5 and 5.0) of these samples evoked a background increase in fluorescence. Additionally, it was observed that a biphasic response for CCR#9 and CCR#10 at the highest (10 µM) concentration.

The results are shown in Figure 5. The general protocol is provided below.

**Cell Culture:** The CCR5 transfected CHO-K1 cell line was grown in MEMα supplemented with 10% FBS (heat inactivated) and 500µg/mL of Hygromycin in a 37°C incubator with 5% CO<sub>2</sub>. When the cells reached 80-90% confluence, they were split at a ratio 1 to 6. CHO-K1 cell lines were plated before the calcium assay as follows: Flasks containing CCR5-CHO-K1 cell line were washed with HBSS/20mM Hepes, 0.05% Trypsin-0.05% EDTA-HBSS (pre-heated at 37°C) is then added to each flask and placed in a 37°C incubator with 5% CO<sub>2</sub> until cells have detached (approximately 1 minute). After the cells detach from the flask, they were pipetted several times to obtain single cells. Media was then added to the cells before spinning down at 1000x g for 7 min.

After the spin, the media was discarded and the cell pellet was resuspended in media. A cell count was performed, as well as a viability check with Trypan Blue. 96 well plates are seeded at 50,000 cells per well per 100µL of media. The plates are incubated for 24 hrs prior to the start of the assay.

**Compounds:** Dilutions – The NK compounds were diluted in 1X Reagent Buffer at 2X the final assay concentration.

**Calcium Assay on the FLEXStation:** The FLEXstation (MDS Pharma, Canada) was calibrated and the temperature was set at 24°C (room temperature). 100µL of loading buffer was added per well and the assay plates were incubated for 1h at 37°C. After incubation, the assay plate was transferred to the FLEXstation where Nonakine analog compound is added and data is acquired. The addition of 100µL of the compound was done per column, through the entire plate, in the FLEXstation unit. Readings were taken every two seconds for a period of 120 seconds. The FLEXstation was set at 24°C and was calibrated with an excitation of 485nm and emission of 525nm, and a cut off of 515nm. The calcium flux data was then acquired and analyzed.

#### **CCR1 & CCR3 Calcium Flux Single Transfectant Assays**

The NK analogs were prepared for screening utilizing recombinant cell lines optimized for the testing of functional responses of G protein-coupled receptors (GPCR) for calcium flux. Upon stimulation of the receptor and increase in intracellular calcium, a photoprotein binds calcium ions and oxidizes the added substrate, coelenterazine with production of CO<sub>2</sub> and emission of a flash of light. For antagonist screening, the recombinant cells were preloaded with NK analog compounds onto a well plate. A pre-incubation period was entered with variable lengths of time and an agonist to the receptor was injected at fixed concentrations into all of the wells. Light emission was recorded over 20 sections to measure the efficacy of the test NK analog compounds as the antagonist was proportional to the inhibition of emitted light. Calcium flux was then measure between the NK analogs. The results are shown in Figures 6 and 7.

#### **Example 5: Pharmacokinetics**

The pharmacokinetics (PK) properties of NK 3, NK 10 and NK 11 were studied following intravenous (IV) and subcutaneous (SC) injections in normal Sprague Dawley (SD) rats. Animals were divided into 6 groups and dosed with single IV or SC injection of NK 3, NK 10 and NK 11. Blood samples were collected up to 72 hr after dosing and analyzed for drug concentrations using ELISA. All animals were healthy during the study. The analogs were dosed at various concentrations over various groups the SD rats, with the results summarized below and depicted in Table 6 and Figure 8 for a 1 mg/kg dose (protein concentration).

Following IV dosing, all three compounds showed bi-phasic concentration declines (Figure 8). In comparison, NK 10 showed the highest clearance (CL) value (367 ml/hr/kg), whereas NK 11 (73 ml/hr/kg) and NK 3 (98 ml/hr/kg) were comparable. NK 10 also showed the longest apparent terminal half live ( $T_{1/2}$ ) for the three compounds tested (i.e., 18 hr vs. 3.4 hr for NK 11 and 2.8 hr for NK 3). However, no significant difference between mean residence time (MRT) was observed (2.8 hr vs. 2.3 hr for NK 11 vs. 1.0 hr for NK 3).

Following SC injections, the peak serum concentrations ( $C_{max}$ ) were similar for the three compounds studied (Table 6 and Figure 8). Compound NK 3, NK 11 and NK 10 showed  $C_{max}$  of 2.1, 1.4, and 1.3  $\mu$ g/mL, respectively. Serum exposures

(AUC) were also comparable (Table 6). Bioavailability for NK 3, NK 11 and NK 10 were 21%, 10%, and 47%, respectively. The higher bioavailability of NK 10 did not result in a higher exposure (AUC), due to its higher CL as compared to the other two compounds.

**Table 6: PK parameters**

Compd	Route	C <sub>max</sub> pg/ml	T <sub>max</sub> hr	AUC hr*pg/ml	Cl/F ml/hr/kg	Vz/F ml/kg	T <sub>1/2</sub> hr	MRT hr	F %
NK 3	IV	10320000	0.08	10173245	98.3	392.9	2.8	1.0	
NK 3	SC	145772	8.00	2109813	474.0	2033.0	3.0	7.0	21
NK 11	IV	12065000	0.25	13680612	73.1	358.4	3.4	2.3	
NK 11	SC	105051	2.00	1435796	696.5	10265.7	10.2	8.7	10
NK 10	IV	8318500	0.08	2724921	367.0	9552.2	18.0	2.8	
NK 10	SC	145232	2.00	1280707	780.8	21101.1	18.7	7.1	47



### **Statement on Language, Scope and Equivalents**

It is to be understood that the invention described herein throughout is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein throughout is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either both of those included limits are also included in the invention.

Unless defined otherwise, all technical and scientific terms used herein throughout have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein throughout can also be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein throughout are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

It must be noted that as used herein throughout and in the appended claims, the singular forms “a”, “and”, and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “Rantes derivative” includes a plurality of such Rantes derivatives and reference to “the Rantes derivative” includes reference to one or more Rantes derivative and equivalents thereof known to those skilled in the art, and so forth.

The publications discussed herein throughout are provided solely for their disclosure prior to the filing date of the present application. Nothing herein throughout is to be construed as an admission that the present invention is not

entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

**CLAIMS**

What is claimed is:

**COMPOSITION**

1. A synthetic chemokine comprising a chemokine polypeptide chain having an N-terminus and a C-terminus, said chemokine polypeptide chain comprising (i) an amino acid sequence and cysteine pattern corresponding to a wild type chemokine, and (ii) a C-terminal truncation relative to said wild type chemokine.
2. The synthetic chemokine of claim 1, wherein said N-terminus comprises amino acids of said chemokine polypeptide chain that are N-terminal to the first disulfide forming cysteine of said chemokine polypeptide chain.
3. The synthetic chemokine of claim 1, wherein said C-terminus comprises amino acids of said chemokine polypeptide chain that are C-terminal to the last disulfide forming cysteine of said chemokine polypeptide chain.
4. The synthetic chemokine of claim 3, wherein said C-terminus comprises a core helix region, and said truncation is C-terminal to said core helix region.
5. The synthetic chemokine of claim 1, wherein said truncation comprises a deletion of one or more amino acid residues having a polar or charged side chain relative to said wild type chemokine.
6. The synthetic chemokine of claim 5, wherein said amino acid residues having a polar or charged side chain are selected from arginine, lysine, aspartic acid, and glutamic acid.

7. The synthetic chemokine of claim 5, wherein said synthetic chemokine is in an oligomeric state consisting substantially of a monomer.
8. The synthetic chemokine of claim 5, wherein said synthetic chemokine is in an oligomeric state consisting substantially of a dimer.
9. The synthetic chemokine of claim 1, wherein said chemokine polypeptide chain comprises one or more amino acid residues that differ from an amino acid residue at a corresponding position in said wild type chemokine.
10. The synthetic chemokine of claim 9, wherein said chemokine polypeptide chain is modified at its C-terminus with one or more amino acid residues that differ from an amino acid residue at a corresponding position in said wild type chemokine.
11. The synthetic chemokine of claim 11, wherein said C-terminus is capped with an amino acid of the formula  $-\text{NH}_2\text{-CH(R)-C(O)-NH}_2$ , where R is an amino acid side chain that is the same or different from the side chain of the amino acid in said wild type chemokine.
12. The synthetic chemokine of claim 9, wherein said chemokine polypeptide chain is modified at its N-terminus with one or more amino acid residues that differ from an amino acid residue at a corresponding position in said wild type chemokine.
13. The synthetic chemokine of claim 12, wherein said chemokine polypeptide chain is modified at its N-terminus with a hydrophobic aliphatic chain.
14. The synthetic chemokine of claim 13, wherein said N-terminus is capped with an amino acid of the formula  $\text{J-X-NH}_2\text{-CH(R)-C(O)-}$ , where R is an amino acid side

chain that is the same or different from the side chain of the amino acid in said wild type chemokine, X is a linker, and J- is said hydrophobic aliphatic chain.

15. The synthetic chemokine of claim 14, wherein X comprises an amino acid derivative.

16. The synthetic chemokine of claim 14, wherein J comprises the formula  $\text{CH}_2\text{-(CH}_2\text{)}_n\text{-}$ , where  $n = 0$  to 20.

17. The synthetic chemokine of claim 1, wherein said chemokine polypeptide chain is covalently modified with one or more polymers.

18. The synthetic chemokine of claim 17, wherein said polymer is linear.

19. The synthetic chemokine of claim 17, wherein said polymer is branched.

20. The synthetic chemokine of claim 17, wherein said polymer comprises an ethylene oxide repeat unit of the formula  $\text{-CH}_2\text{-CH}_2\text{-O-}$ .

21. The synthetic chemokine of claim 20, wherein said polymer comprises polyethylene glycol.

22. The synthetic chemokine of claim 20, wherein said polymer comprises a polyamide.

23. The synthetic chemokine of claim 17, wherein said polymer comprises a fatty acid.

24. The synthetic chemokine of claim 1, wherein said chemokine polypeptide chain comprises an amino acid sequence that is substantially homologous to the amino acid sequence of said wild type chemokine.
25. The synthetic chemokine of claim 24, wherein said wild type chemokine is a CC Chemokine.
26. The synthetic chemokine of claim 24, wherein said wild type chemokine is a CXC Chemokine.
27. The synthetic chemokine of claim 1, wherein said wild type chemokine is selected from the group consisting of Rantes, MIP1 $\alpha$ , MIP1 $\beta$ , or MCP-1.
29. The synthetic chemokine of claim 1, wherein said chemokine polypeptide chain comprises an amino acid sequence of a SEQ ID NO:1.
30. A composition comprising a synthetic chemokine having an amino acid sequence depicted in SEQ ID NO. 1.
31. A composition comprising a synthetic chemokine selected from the group consisting of NK1, NK2, NK3, NK4, NK5, NK6, NK7, NK8, NK9, NK10, NK11, NK12 and NK13.
32. The synthetic chemokine of claim 1, wherein said chemokine polypeptide chain is produced by chemical synthesis.

33. The synthetic chemokine of claim 32, wherein said chemical synthesis comprises the chemoselective ligation of non-overlapping peptide segments of said chemokine polypeptide chain.
34. The synthetic chemokine of claim 32, wherein said chemoselective ligation is native chemical ligation.
35. A pharmaceutical composition comprising a synthetic chemokine according to any one of claims 1, 30 and 31, or pharmaceutically acceptable salts thereof.
36. The pharmaceutical composition according to claim 35, which comprises one or more excipients selected from the group consisting of a buffer, a carrier protein, an amino acid, a detergent, a lipid, a water-soluble polymer, and a preservative.
37. The pharmaceutical composition according to claim 35, which comprises one or more additional bioactive agents other than said synthetic chemokine.
38. A method of treating a disease state in mammals that is alleviated by treatment with a chemokine receptor antagonist, said method comprising administering to a mammal in need of such a treatment a therapeutically effective amount of a synthetic chemokine according to any one of claims 1, 30, and 31.
39. The method of claim 38, wherein chemokine receptor is down regulated as a result of binding of said synthetic chemokine to said chemokine receptor.
40. The method of claim 38, wherein the mammal has a disorder selected from the group consisting of AIDS, psoriasis, multiple sclerosis, cancer, asthma, allergic rhinitis, atopic dermatitis, atheroma, atherosclerosis, or rheumatoid arthritis.

41. The method of claim 40, wherein said disorder in the mammal is incident to a therapy selected from the group consisting of antiviral therapy, psoriasis therapy, multiple sclerosis therapy, cancer chemotherapy, asthma therapy, allergic rhinitis therapy, atopic dermatitis therapy, atheroma therapy, atherosclerosis therapy, and rheumatoid arthritis therapy.

42. The method of claim 41, wherein the synthetic chemokine is administered before, concurrently with, or after said therapy.

43. A method of producing a synthetic chemokine in a substantially purified oligomeric form, said method comprising:

synthesizing a protein pool containing a synthetic chemokine protein comprising a chemokine polypeptide chain having an N-terminus and a C-terminus, said chemokine polypeptide chain comprising (i) an amino acid sequence and cysteine pattern corresponding to a wild type chemokine, and (ii) a C-terminal truncation relative to said wild type chemokine; and

purifying from said protein pool one or more oligomeric forms of said synthetic chemokine protein so as to produce a synthetic chemokine in a substantially single oligomeric form.

44. The method of claim 43, wherein said single oligomeric form is selected from the group consisting of monomer and dimer.

45. The method of claim 43, wherein said synthetic chemokine protein is covalently modified with one or more polymers.

46. The method of claim 43, wherein said synthesizing comprises chemical synthesis.



47. The method of claim 46, wherein said chemical synthesis comprises the chemoselective ligation of non-overlapping peptide segments of said chemokine polypeptide derivative.

48. The method of claim 47, wherein said chemoselective ligation is native chemical ligation.

49. A kit comprising in a container a chemokine polypeptide derivative of any one of claims 1, 30 and 31.

**CHEMOKINE DERIVATIVES, METHODS OF MANUFACTURE, AND USES****ABSTRACT OF THE DISCLOSURE**

The invention relates to synthetic chemokines, methods of manufacture and uses thereof. The synthetic chemokines of the invention include one or more amino acid deletions at the C-terminus relative to the corresponding wild type chemokine, which also can include one or more covalently attached polymers, as well one or more additional amino acid changes or chemical modifications. The invention also provides synthetic chemokines in a substantially purified oligomeric form, such as in a monomer or dimer form. Further provided are methods for synthesizing the synthetic chemokines of the invention, pharmaceutical formulations and kits thereof, and their use as research tools and medicaments.

FIG.1

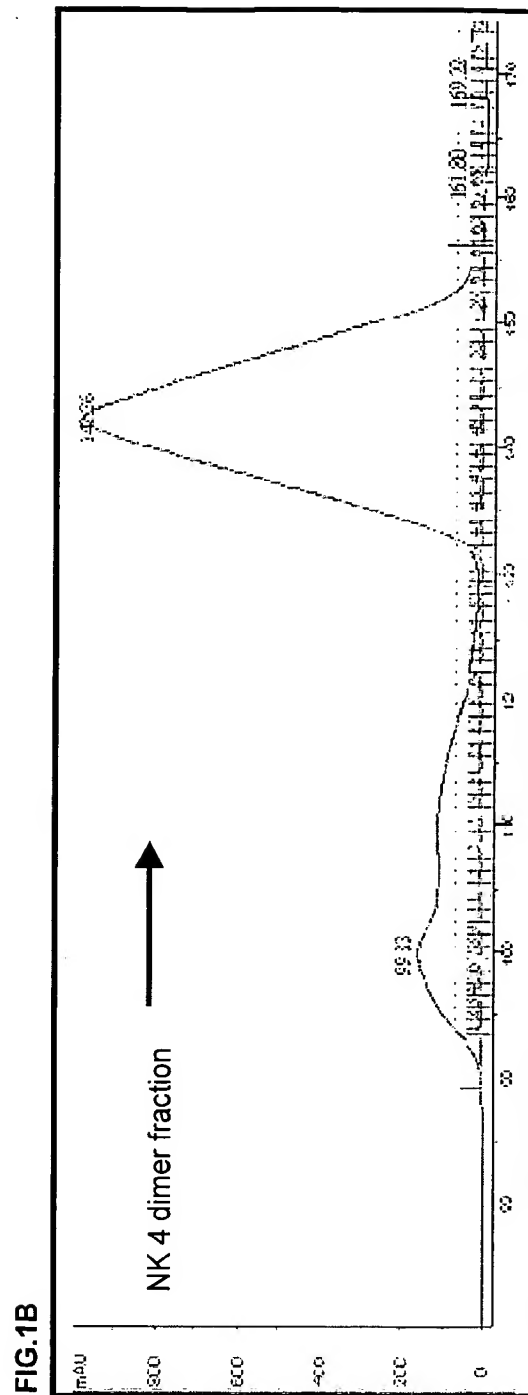
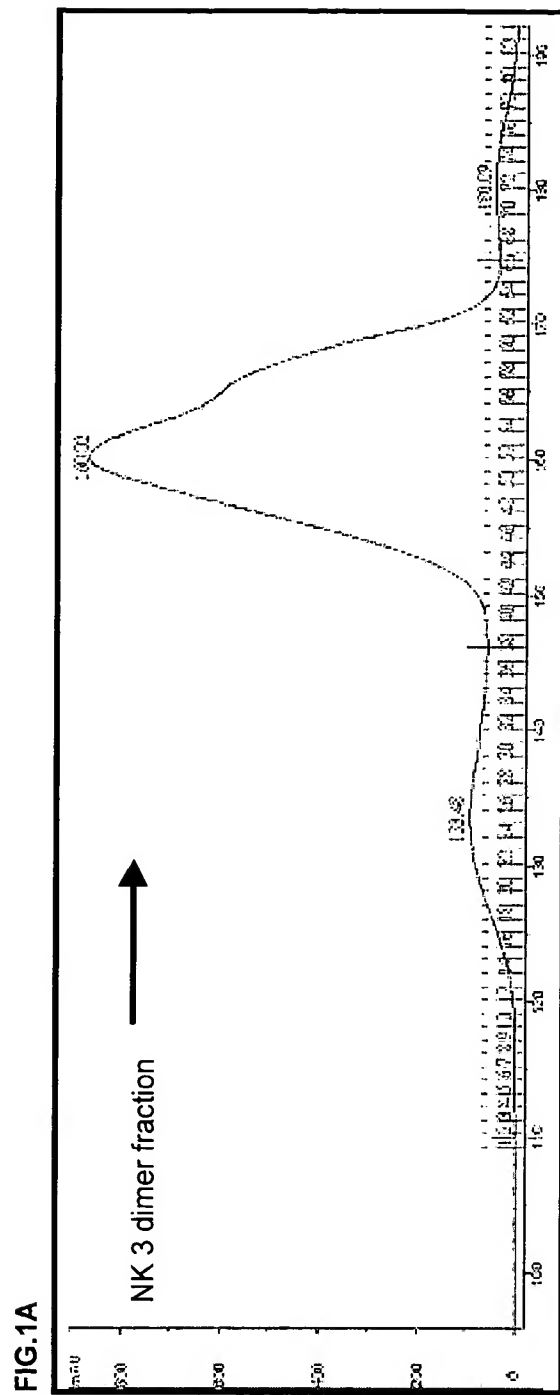


FIG.2

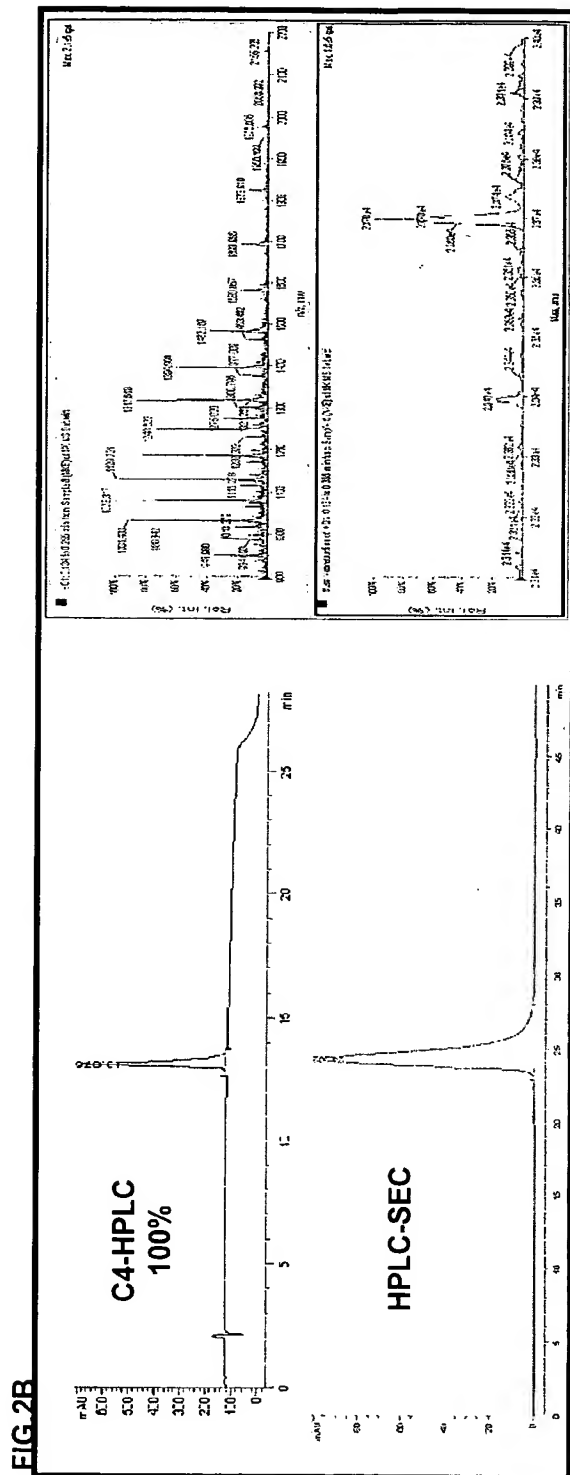
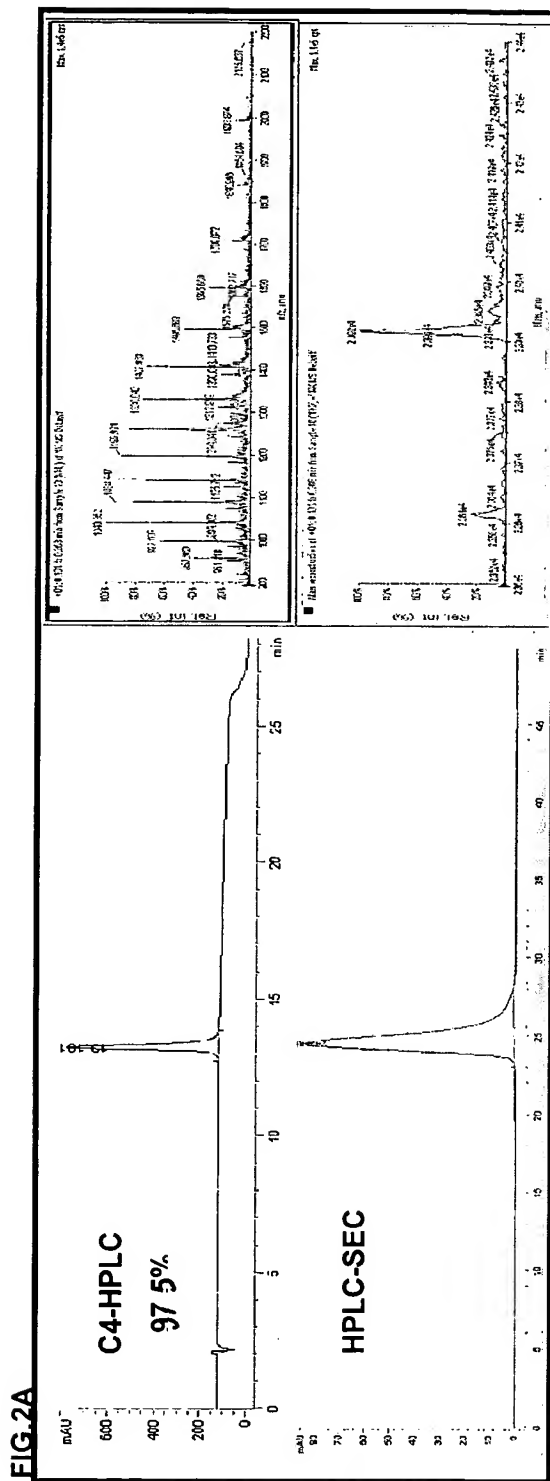
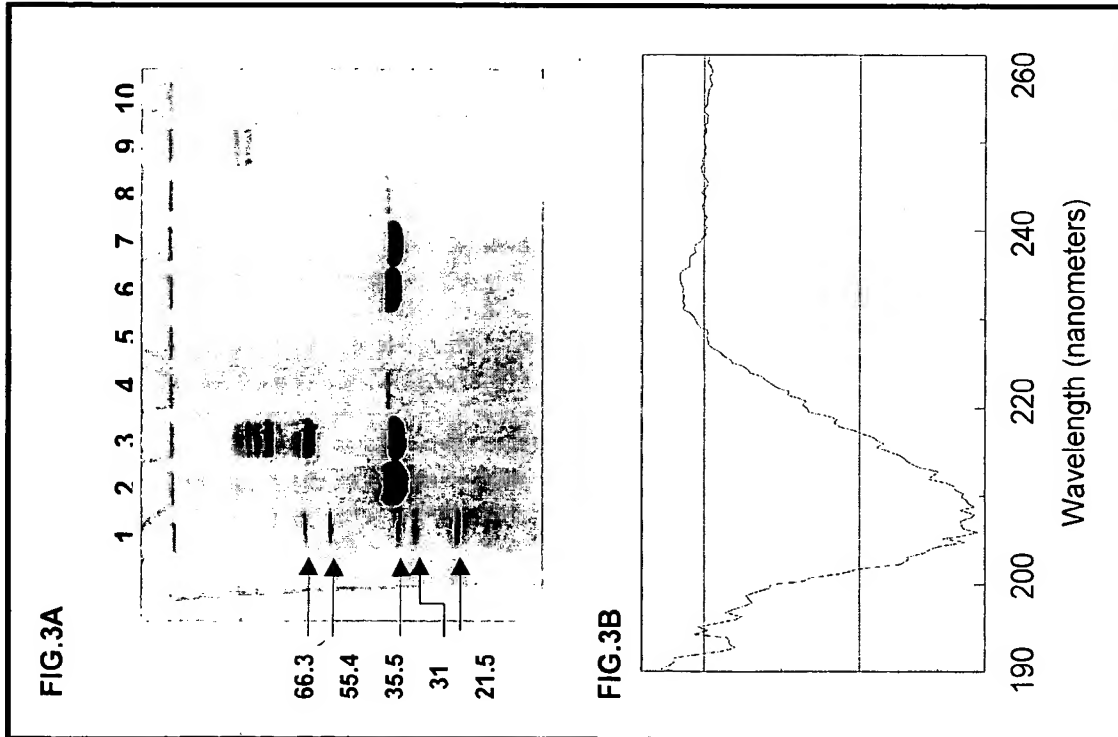
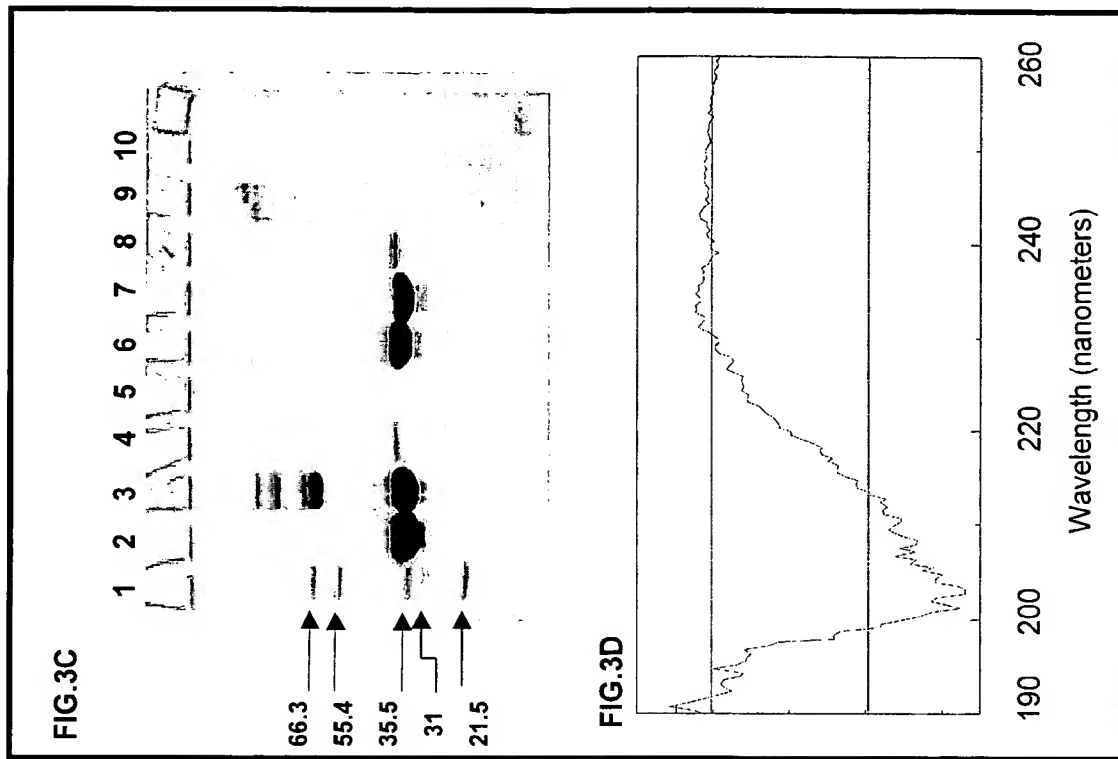


FIG.3



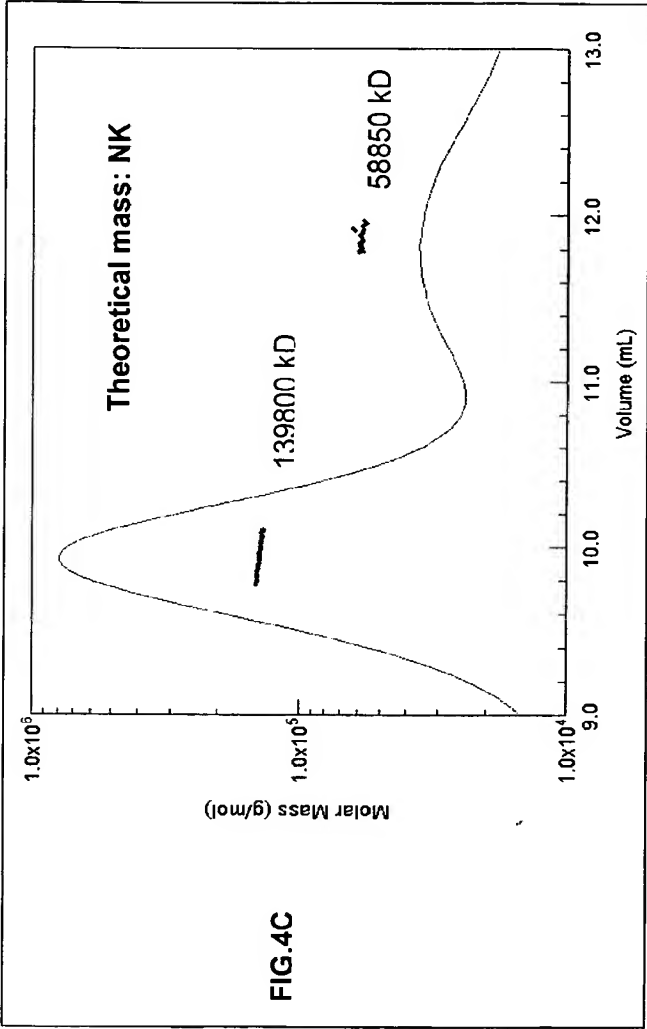
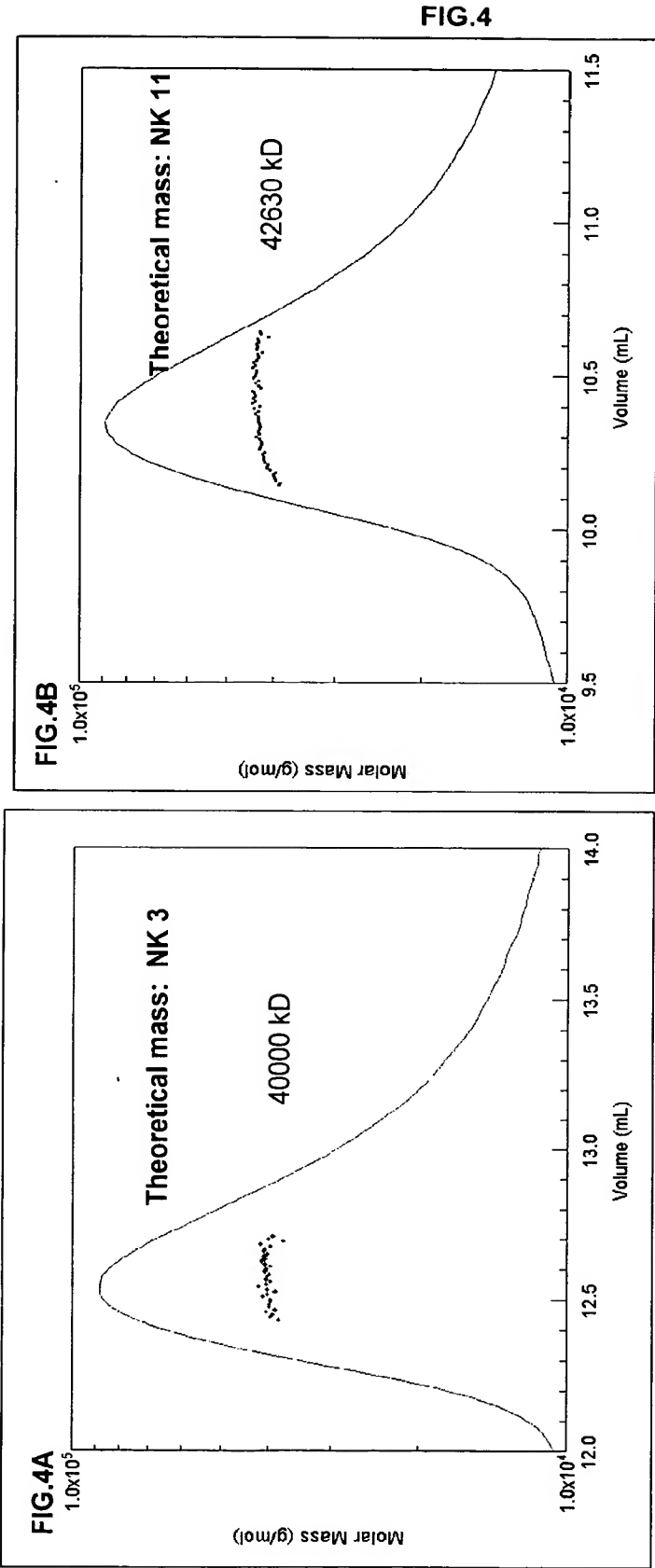


FIG.5

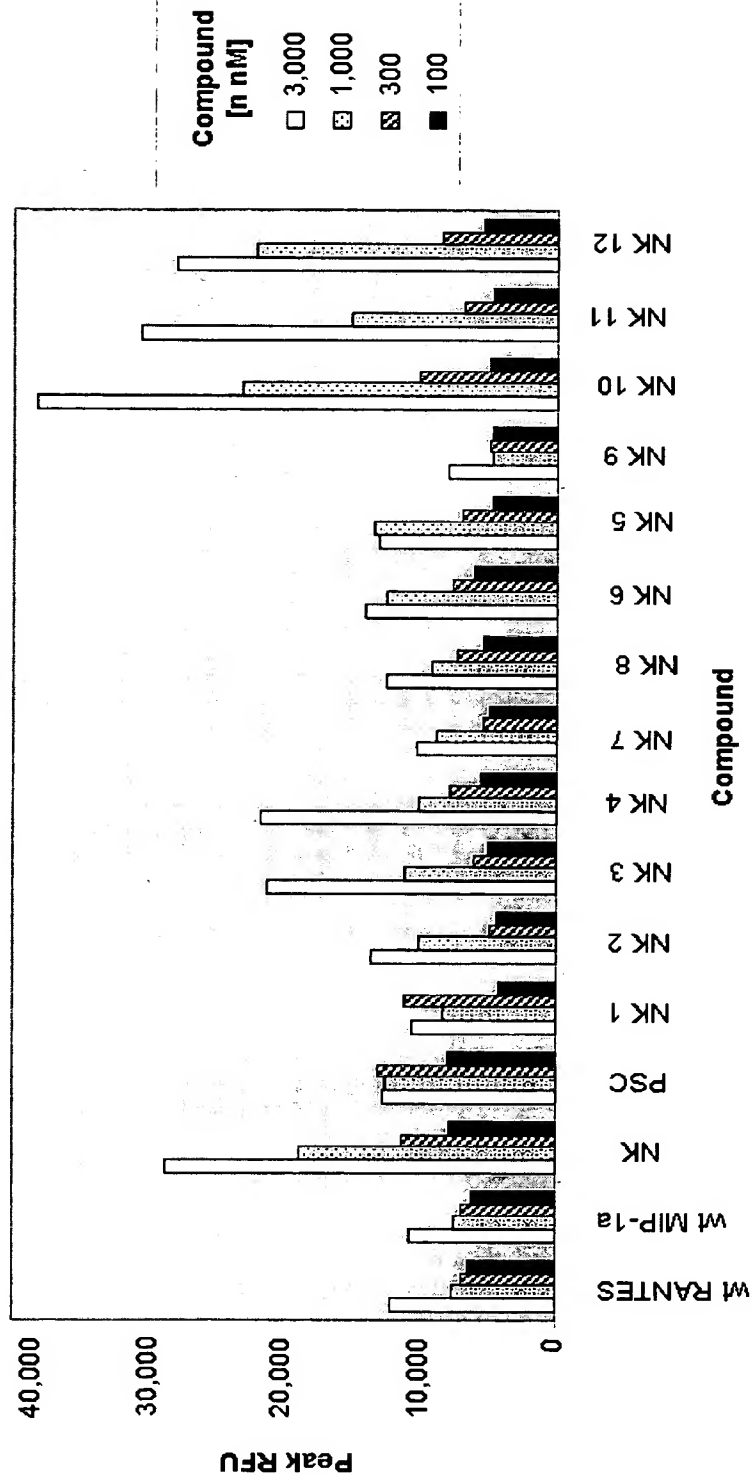


FIG.6

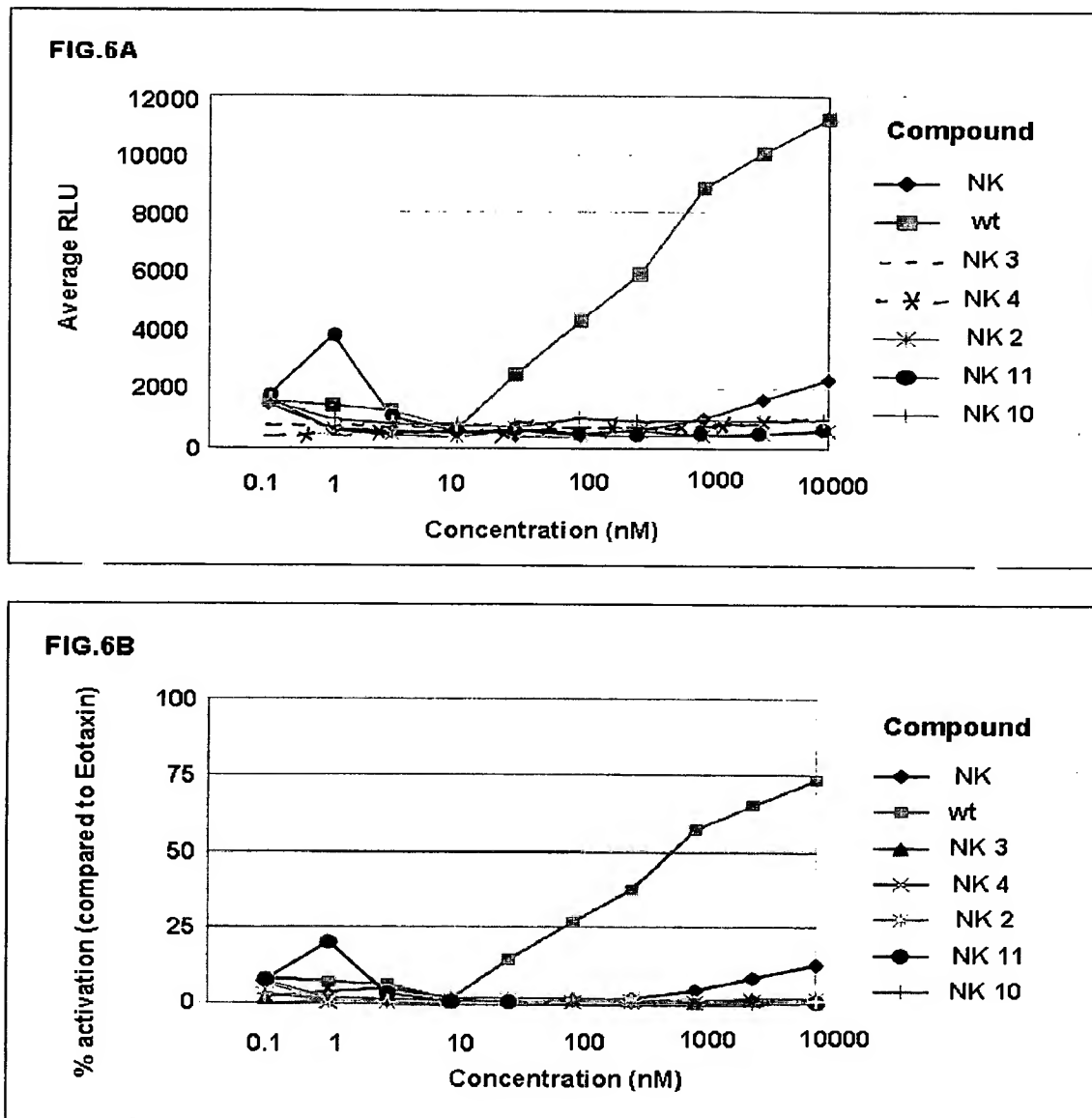
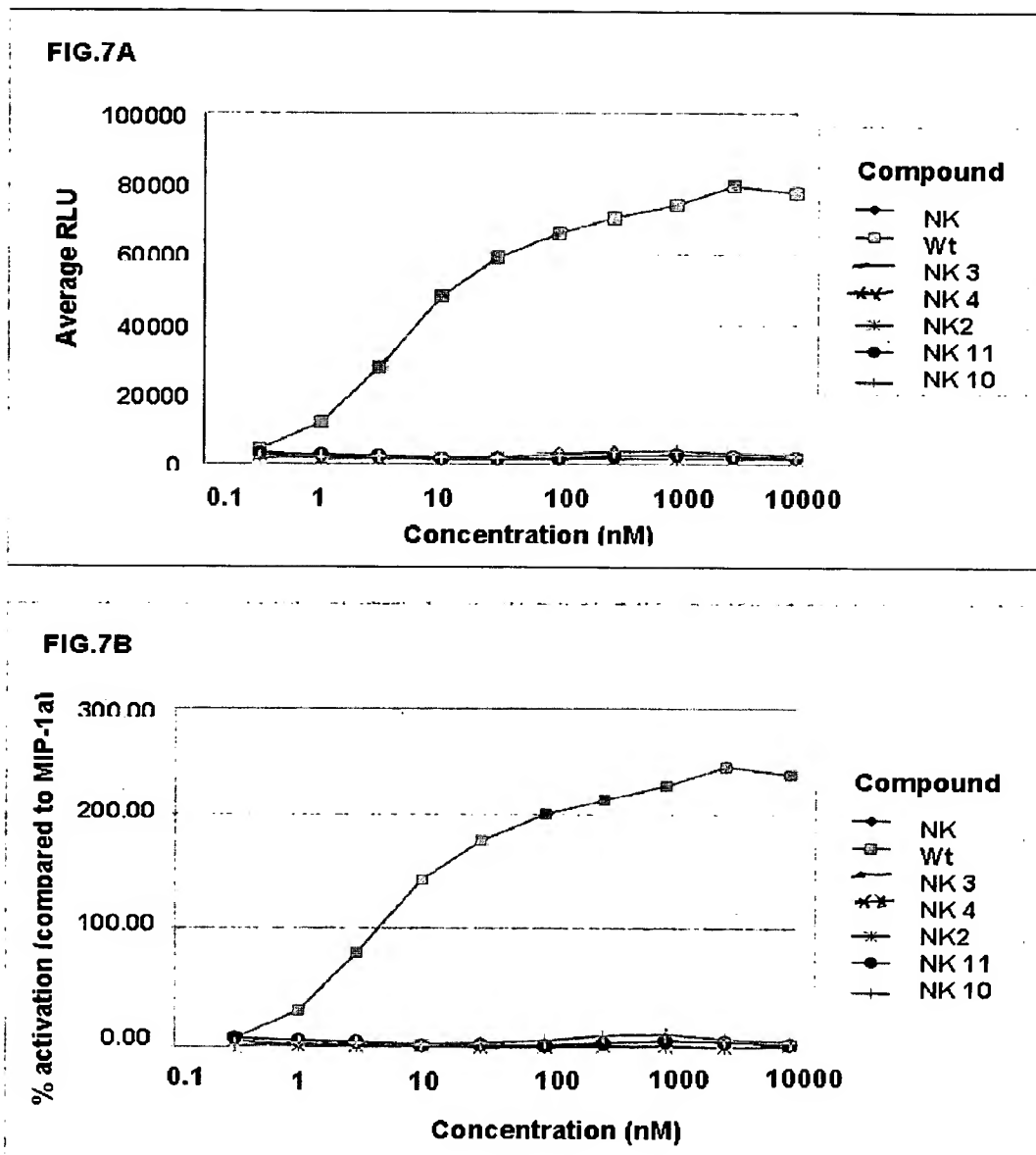




FIG.7



Patent

Atty. Dkt. No.: GRFN-060/00US PRV

FIG.8

